Course :	ELE BOT 242		Credit:	3(1+2)	Semester-IV
Course title:	Micro-propagation Technologies				

Syllabus

Theory

Introduction, History, Advantages and limitations; Types of cultures (seed, embryo, organ, callus, cell), Stages of micropropagation, Axillary bud proliferation (Shoot tip and meristem culture, bud culture), Organogenesis (callus and direct organ formation), Somatic embryogenesis, cell suspension cultures, Production of secondary metabolites, Somaclonal variation, Cryopreservation

Practical

Identification and use of equipments in tissue culture Laboratory, Nutrition media composition, sterilization techniques for media, containers and small instruments, sterilization techniques for explants, Preparation of stocks and working solution, Preparation of working medium, Culturing of explants:Seeds, shoot tip and single node, Callus induction, Induction of somatic embryos regeneration of whole plants from different explants, Hardening procedures.

Teaching Schedules

a) Theory

Lecture	Topic	Weightages (%)
1 & 2	Meaning and concept of *in vitro* culture and micro-propagation, Historical milestones.	05
3	Tissue culture methodology: Sterile techniques	10
4	Synthetic and natural media components, growth regulators, environmental requirement.	10
5	Totipotency, dedifferentiation; genetic control of regeneration;	05
6	Plant regeneration pathways - Organogenesis and Somatic embryogenesis;	10
7	Organogenesis- Purpose, methods and requirements for organogenesis, indirect and direct organogenesis;	10
8, 9 & 10	Somatic embryogenesis- Procedures and requirements for organogenesis, indirect and direct embryogenesis; Differences between somatic and gametic embryogenesis,	15
11, 12 & 13	Micro-propagation- Definition, methods, stages of micro-propagation and its significance; Advancement and future prospects of micro-propagation.	20
14 & 15	Micropropagation - Axillary bud proliferation approach- Shoot tip and meristem culture;	10
16	Synthetic seed- Concepts, necessity, procedure and requirements for production of synthetic seeds.	5
	Total	**100**

b) Practical

Experiment	Topic
1	Laboratory organization of Plant Tissue Culture Laboratory
2	Safety Measures in Laboratory
3, 4, 5	Sterilization techniques: Common Contaminant in Laboratory, Sterilization of glassware, Working of Laminar air flow cabinet

6, 7	Culture Media: Definition, Components of Media, Stock Solution, Working Solution, Sterilization of Media.
8, 9	Preparation and sterilization of growth regulators/thermolabile compounds.
10,11	Preparation of working medium
12,13	Experimentation on determining optimum concentration of growth regulators.
14	Sterilization techniques for explants.
15, 16, 17, 18	Callus induction from different parts of plants
19, 20, 21, 22	Regeneration of whole plants from induced callus using different parts of plants.
23, 24	Induction of somatic embryos.
25, 26	Experiments of synthetic seeds production and testing storability and germination efficiency.
27, 28, 29, 30, 31 ,32	Direct regeneration into whole plants using bud, node and other tissues.

Suggested readings:

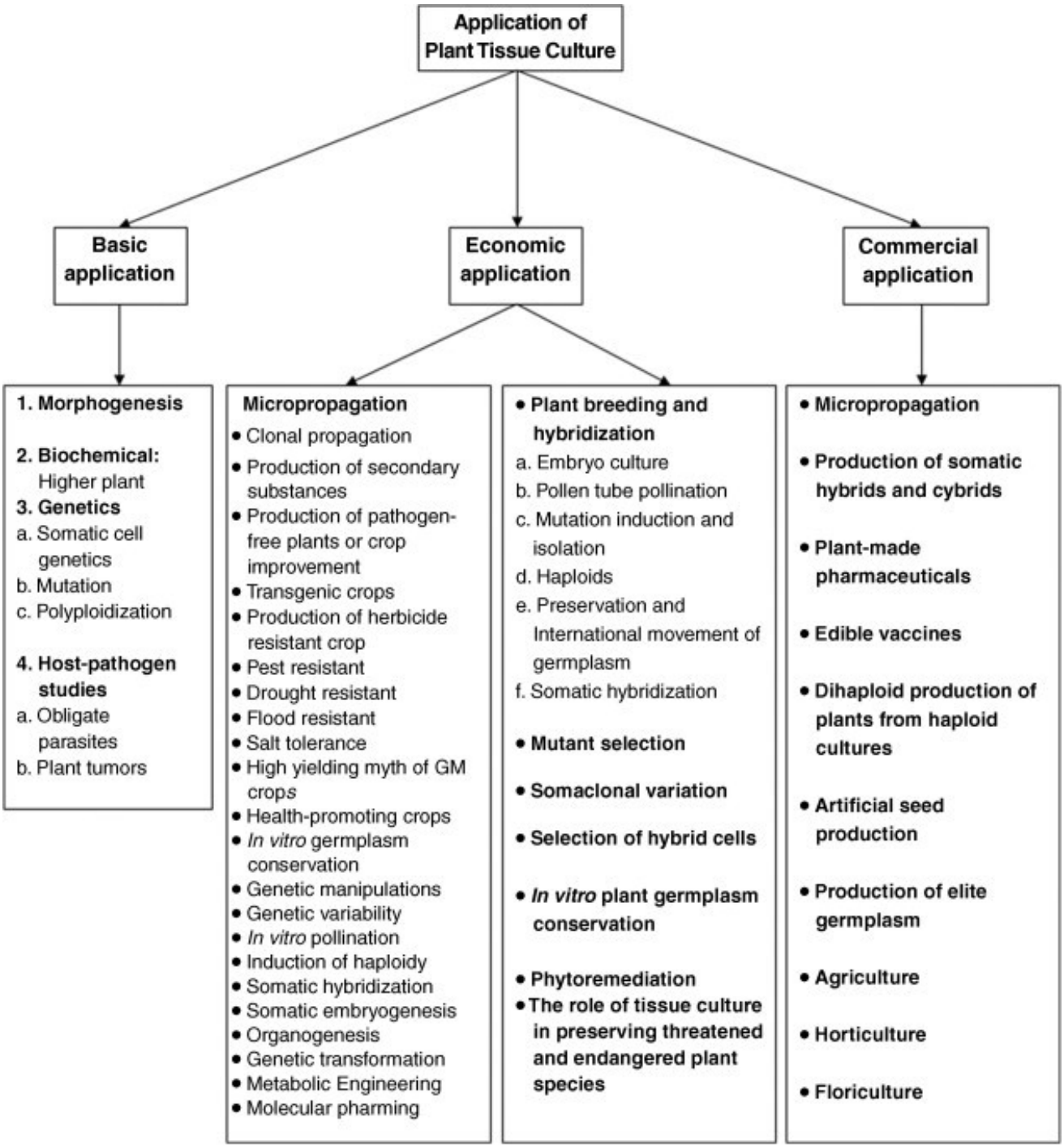
1) Plants from Test Tubes: An introduction to Micropropagation (Fourth Edition) – Lydiane Kyte, John Kleyn, Holly Scoggins and Mark Bridgen (Timber Press)
2) Introduction top Plant Tissue Culture- M. K. Razdan (Science Publisher)
3) Somatic Embryogenesis: Fundamental Aspects and Application – Loyola-Vargas, Victor, Ochoa-Aleja, Neftali (Springer)
4) Plant Tissue Culture, Techniques and Experiment – Robert H Smith (AP)
5) Plant Tissue Culture- Protocols in Plant Biotechnology - M.C. Gayatri and R. Kavyashree (Narosa Publishing)
6) Practical biotechnology and Plant Tissue Culture- Prof. Santosh Nagar, Dr. Madhavi Adhav (S Chand)

Lecture 01

Meaning and concept of *in vitro* culture and micro-propagation, Historical milestones.

Meaning of Plant Tissue Culture:

Plant tissue culture is the technique of maintaining and growing plant cells, tissues or organs especially on artificial medium in suitable containers under controlled environmental conditions.e The part which is cultured is called explant, i.e., any part of a plant taken out and grown in a test tube, under sterile conditions in special nutrient media. This capacity to generate a whole plant from any cell/explant is called cellular toti-potency. In fact, the whole plant can be regenerated from any plant part (referred to as explant) or cells. Gottlieb Haberlandt first initiated tissue culture technique in 1902.



Growing any part of the plant (explants) like, cells, tissues and organs, in an artificial medium under controlled conditions (aseptic conditions) for obtaining large scale plant propagation is called micropropagation.

The basic concept of micropropagation is the plasticity, totipotency, differentiation, dedifferentiation and redifferentiation, which provide the better understanding of the plant cell culture and regeneration. Plants, due to their long life span, have the ability to withhold the extremes of conditions unlike animals.

The plasticity allows plants to alter their metabolism, growth and development to best suit their environment. When plant cells and tissues are cultured *in vitro* , they generally exhibit a very high degree of **plasticity**, which allows one type of tissue or organ to be initiated from another type. Hence, whole plants can be subsequently regenerated and this regenerated whole plant has the capability to express the total genetic potential of the parent plant. This is unique feature of plant cells and is not seen in animals. Unlike animals, where differentiation is generally irreversible, in plants even highly mature and differentiated cells retain the ability to regress to a meristematic state as long as they have an intact membrane system and a viable nucleus.

The meristematic tissues are differentiated into simple or complex tissues called **differentiation**. Reversion of mature tissues into meristematic state leading to the formation of callus is called **dedifferentiation**.

The ability of callus to develop into shoots or roots or embryoid is called **redifferentiation**.

The inherent potentiality of a plant cell to give rise to entire plant and its capacity is often retained even after the cell has undergone final differentiation in the plant system is described as cellular **totipotency**.

Terms commonly used in plant tissue culture

Plasticity and totipotency: Plants due to their long-life cycle and lack of mobility have developed greater capacity to survive extreme conditions. Many of the processes involved in plant growth and development adapt to environmental conditions. This plasticity allows plants to alter their metabolism, growth and development to best suit their environment. Plants also have, in many cases, the ability to regenerate lost organs (roots, shoots etc.). Cell division can be induced from almost all plant tissues. When plant cells and tissues are cultured in vitro, they generally exhibit a very high degree of plasticity, which allows one type of tissues or organs to be initiated from another type under the influence of chemical stimuli. In this way, whole plants can be subsequently regenerated. For example, embryos may be developed in vitro from somatic cells and haploid cells, as well as from normal zygote and all these, in turn, could develop into whole plants.

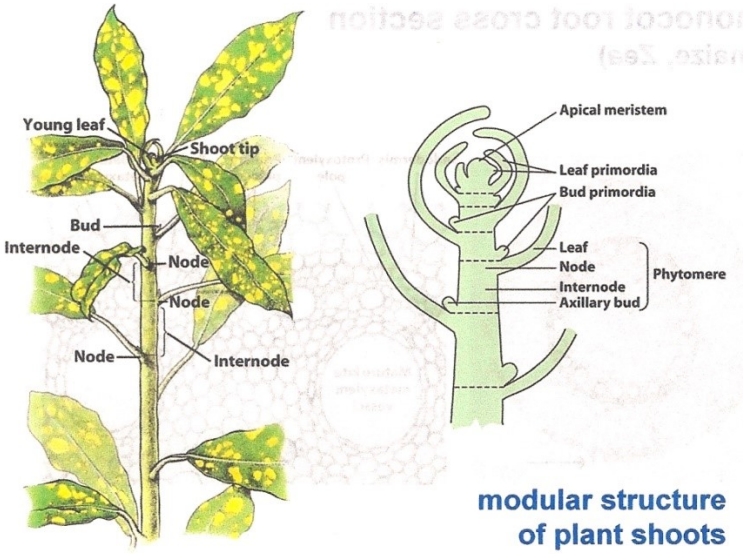
Totipotency is the potentiality or property of a cell to produce a whole organism or whole parent plant in the presence of correct physical and chemical stimulus.

Recalcitrant: The opposite of totipotency is recalcitrant. An explant is said to be recalcitrant if it is difficult to give rise to organism or plant.

Culture: Growing cells, tissues, plant organs, or whole plants in nutrient medium, under aseptic conditions e.g. cell culture, embryo culture, shoot-tip culture, anther culture.

Contaminants: In tissue culture it refers to the micro-organisms (Bacteria, Fungi), which may inhibit the growth of cells or tissues in culture.

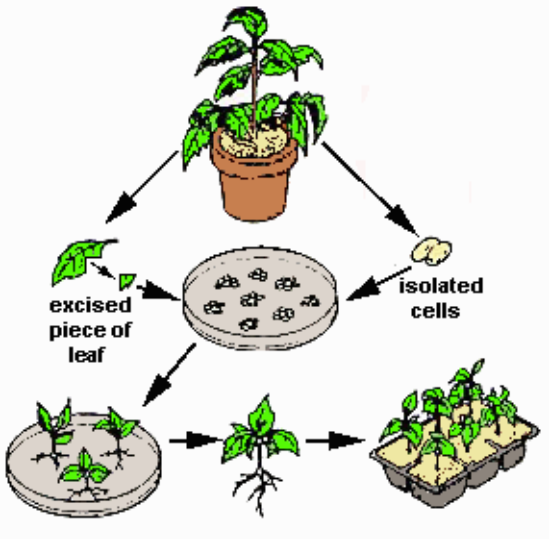
Explant: A plant organ or piece of tissue used to initiate a culture.



Node: A region on the stem from which a leaf bearing an axillary bud arises.
Morphogenesis: The anatomical and physiological events involved in the growth and development of an organism resulting in the formation of its characteristic organ and structures, or in regeneration.
Meristem: A localized group of actively dividing cells, from which permanent tissue system i.e. root, shoot, leaf and flower are derived. Apical meristem is located at the apices of main and lateral shoots.
Meristematic: having the characteristics of a meristem, especially high mitotic activity.
Meristemoid: A localized group of meristematic cells that arise in the callus and may give rise to roots and or shoots.
Regeneration: In tissue culture, a morphogenetic response that results in the formation of new organs, embryos or whole plants from cultured explants.
Dedifferentiation: The phenomenon of mature cells reverting to meristematic state to produce callus is dedifferentiation. Dedifferentition is possible because the non-dividing quiescent cells of the explant, when grown in a suitable culture medium revert to meristematic state.
Redifferentiation: The ability of the callus cells to differentiate into a plant organ or a whole plant is regarded as redifferentiation.
Adventitious: Developing from unusual points of origin, such as shoots or roots arising from a leaf or stem tissues other than the axils or apex; and embryos from any cell other than the zygote.
Determined: Cells that are committed to a particular pathway of development or differentiation but which have yet to overtly express this pathway.
Heterotrophic: Dependent on an external energy source; not self-reliant compare to autotrophic.
Ploidy: Term used to describe the number of genomes present in the nucleus of a cell or plant.

Lecture 02
Plant tissue culture and its history

Plant tissue culture broadly refers to the cultivation *in vitro* of all plant parts, whether a single cell, a tissue or organ under aseptic conditions. Recent progress in the field of plant tissue culture made this area as one of the most dynamic and promising experimental biology.



Overview of tissue culture process

This new technique has enabled us to increase the knowledge in the following field of studies.

- Totipotency, nutrition, metabolism, division, differentiation and preservation of plant cells.
- Morphogenesis and plant regeneration from individual cells or tissues through the process namely organogenesis or somatic embryogenesis.
- Variations generated through *in vitro* culture.
- Evolution of haploids through anther and pollen culture including ovule culture.
- Wide hybridization programmes through ovule, ovary and embryo cultures to overcome both pre zygotic and post zygotic sterility mechanisms
- Micropropagation of plant materials
- *In vitro* selection of mutants tolerant to biotic and abiotic stresses.
- *In vitro* culture and secondary metabolite biosynthesis.
- Plant genetic engineering through *in vitro* culture methods and DNA transfer technique.

Thus plant cell, tissue and organ culture permeates plant biotechnology and cements together its various aspects like Physiology, Biochemistry, Genetics and Cell Biology.

Like other subjects, plant cell and tissue culture has its own origin and development. The chronology of major events in this field is presented for the benefit of the new entrants into this field.

Historical Milestones in Plant tissue culture

-1902 - Haberlandt proposed concept of in vitro cell culture
- 1904 - Hannig cultured embryos from several cruciferous species

- 1922 - Kolte and Robbins successfully cultured root and stem tips respectively
- 1926 - Went discovered first plant growth hormone –Indole acetic acid
- 1934 - White introduced vitamin B as growth supplement in tissue culture media for tomato root tip
- 1939 - Gautheret, White and Nobecourt established endless proliferation of callus cultures
- 1941 - Overbeek was first to add coconut milk for cell division in Datura
- 1946 - Ball raised whole plants of Lupinus by shoot tip culture
- 1954 - Muir was first to break callus tissues into single cells
- 1955 - Skoog and Miller discovered kinetin as cell division hormone
- 1957 - Skoog and Miller gave concept of hormonal control (auxin: cytokinin) of organ formation
- 1959 - Reinert and Steward regenerated embryos from callus clumps and cell suspension of carrot (Daucus carota)
- 1960 - Cocking was first to isolate protoplast by enzymatic degradation of cell wall
Plant Tissue Culture: Current Status and Opportunities 3
- 1960 - Bergmann filtered cell suspension and isolated single cells by plating
- 1960 - Kanta and Maheshwari developed test tube fertilization technique
- 1962 - Murashige and Skoog developed MS medium with higher salt concentration
- 1964 - Guha and Maheshwari produced first haploid plants from pollen grains of Datura (Anther culture)
- 1966 - Steward demonstrated totipotency by regenerating carrot plants from single cells of tomato
- 1970 - Power et al. successfully achieved protoplast fusion
- 1971 - Takebe et al.regenerated000000 first plants from protoplasts
- 1972 - Carlson produced first interspecific hybrid of Nicotiana tabacum by protoplast
- 1974 – Rein hard introduced biotransformation in plant tissue cultures
- 1977 - Chilton et al. successfully integrated Ti plasmid DNA from Agrobacterium tumefaciens in plants
- 1978- Melchers et al. carried out somatic hybridization of tomato and potato resulting in pomato
- 1981- Larkin and Scowcroft introduced the term somaclonal variation
- 1983 - Pelletier et al.conducted intergeneric cytoplasmic hybridization in Radish and Grape
- 1984 - Horsh et al. developed transgenic tobacco by transformation with Agrobacterium
- 1987 - Klien et al. developed biolistic gene transfer method for plant transformation
- 2005 - Rice genome sequenced under International Rice Genome Sequencing Project.

Types of tissue culture

Callus culture: Callus culture may be defined as production and maintenance of an unorganized mass of proliferative cell from isolated plant cell, tissue or organ by growing them on artificial nutrient medium in glass vials under controlled aseptic conditions.

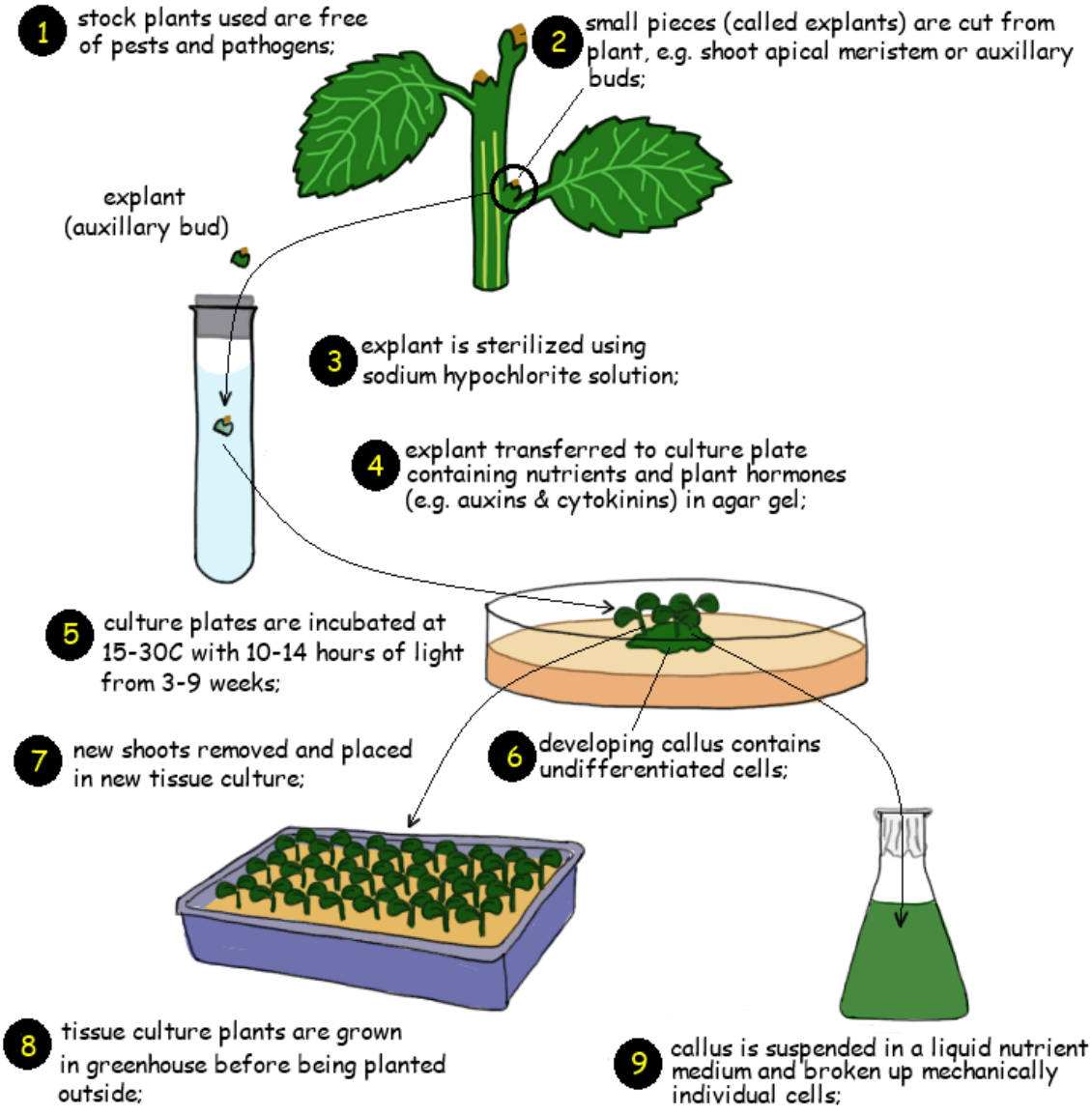
Organ culture: That may allow differentiation and preservation of the architecture. The organ culture refers to the *in vitro* culture and maintenance of an excised organ primordial or whole or part of an organ in way and function.

Single cell culture: Single cell culture is a method of growing isolated single cell aseptically on nutrient medium under controlled condition.

Suspension culture: Suspension culture is a type of culture in which single cell or small aggregates of cell multiply while suspended in agitated liquid medium. Suspension cultures are used in induction of somatic embryos and shoots, production of secondary metabolites, *in vitro* mutagenesis, selection of mutants and genetic transformation studies.

Embryo culture: Embryo culture may be defined as aseptic isolation of embryo (of different developmental stages) from the bulk of maternal tissue of mature seed or capsule and *in vitro* culture under aseptic and controlledphysical condition in glass vials containing nutrient semisolid or liquid medium to grow directly into plantlet.

Anther culture: Androgenesis is the *in vitro* development of haploid plants originating from potent pollen grains through a series of cell division and differentiation.
Pollen culture: Pollen culture is the *in vitro* technique by which the pollengrains (preferably at the microscope stages) are squeezed from the intact anther and then cultured on nutrient medium where the microspores without producing male gametes.
Somatic Embryogenesis: Somatic embryogenesis is the process of a single or group of cells initiating the development pathway that leads to reproducible regeneration of non zygotic embryos capable of germinating to form complete plants.
Protoplast Culture: It is the culture of isolated protoplasts which are naked plant cells surrounded by plasma membrane which is potentially capable of cell wall regeneration, cell division, growth and plant regeneration on suitable medium under aseptic condition
Shoot tip and Meristem culture: The tips of shoots (which contain the shoot apical meristem) can be cultured *in vitro* producing clumps of shoots from either axillary or adventitious buds. This method can, be used for clonal propagation.
Explant Culture: There are variety of forms of seed plants viz., trees, herbs, grasses, which exhibit the basic morphological units i.e. root, stem and leaves. Parenchyma is the most versatile of all types of tissues. They are capable of division and growth.


1 stock plants used are free of pests and pathogens;
2 small pieces (called explants) are cut from plant, e.g. shoot apical meristem or auxillary buds;
explant (auxillary bud)
3 explant is sterilized using sodium hypochlorite solution;
4 explant transferred to culture plate containing nutrients and plant hormones (e.g. auxins & cytokinins) in agar gel;
5 culture plates are incubated at 15-30C with 10-14 hours of light from 3-9 weeks;
6 developing callus contains undifferentiated cells;
7 new shoots removed and placed in new tissue culture;
8 tissue culture plants are grown in greenhouse before being planted outside;
9 callus is suspended in a liquid nutrient medium and broken up mechanically individual cells;

1901- Morgan, T.H coined the term totipotency to describe the capability of a cell to form an individual plant.
1902- Haberlandt, G – Father of plant tissue culture published a paper on "Experiments on the culture of isolated plant cells: In that he says "I should like to point out the fact that, in my cultures, despite the conspicuous growth of the cells which frequently occurred, cell division was never observed. It will be the problem of future culture experiments to discover the condition under which isolated cells undergo division". He clearly set forth the purposes and potentialities of cell culture after having attempted and failed in the culture of isolated plant cells. The reasons for his failure may be (i) use of three monocotyledonous genera for much of his work, (ii) culture of mature differentiated green mesophyll and pallisade tissues,
(iii) contamination during culture growth.

Haberlandt, G – Father of plant tissue culture

1922- Kotte, W succeeded in cultivating small root tips of pea and maize in various nutrients. The roots developed well and their growth was maintained for long periods but no subculture was attempted.
1922- Robbins, W.J started cultivation of excised root tips and stem tips of maize under sterile conditions; however, the cultures did not survive independently.
1925- Liabach, F demonstrated the most important application of the embryo culture by crossing *Linum perenae* with *L. austriacum* to get hybrid plants from shriveled seeds.
1934- Gautheret, R.J made preliminary attempts with liquid medium for cultivating plant tissues but failed completely. Later he cultured the explants on medium solidified with agar, and got healthy calli from the explants.
1934- White, P.R obtained indefinite survival of cultured tomato roots on sub culturing in liquid medium.
1939- White, P.R., Gautheret, R.J. and Nobecourt, P simultaneously announced the possibility of cultivating plant tissues for unlimited periods.
194l- Van Overbeek, J., Conklin, **M.E. and Blackeslee, A.F** established importance of coconut milk for growth and development of very young *Datura* embryos.
1942- White, P.R. and Braun, A.C initiated studies on crown gall and tumour formation in plants.
1944- Skoog, F started his work on organogenesis in tobacco callus.

1946- Ball, E.A showed development of plantlets from sterile cultures of stem tips in *Tropaeolum* and *Lupinus.* **He is considered as father of micropropagation.**

1952- Morel, G. and Martin, C were the first to demonstrate that virus free plants can be recovered from infected plants through shoot meristem culture.

1953- Muir, W.H found out the cultural conditions favouring the isolation and growth of single cells from higher plants *in vitro* and established nurse culture technique.

1955- Miller, C.O., Skoog, F., Von Saltza, M. and Strong, F.M identified a cell division factor *viz*., 6-furfualamino purine commonly called kinetin.
1957- Skoog, F. and Miller, C.O advanced the hypothesis that shoot and root initiation in cultured callus can be regulated by particular ratios of auxins and cytokinin.
1957- Skoog, F. and Miller, C.O discovered and introduced the idea of synergistic effects of auxins and cytokinins in promoting cell division in tobacco.

1958- Steward, F.C., Mapes, M.O. and Mears, K observed the phenomenon of **somatic embryogenesis** in suspension culture of carrot. They also reported that cells in suspension derived from explanted roots of cultivated carrots were capable of forming unorganised cell clusters, which in turn could yield first roots, then shoots and ultimately whole plants.

1959- Reinert, J observed the **somatic embryo** formation from callus cultures of carrot grown on an agarified medium.

1960- Morel, G discovered a technique to produce virus free progenies by meristem culture in *Cymbidium.*

1964- Guha, S. and Maheshwari, S.C cultured mature anthers of *Datura innoxia* to study the physiology of meiosis and accidentally noticed the development of embryoids from the anthers plated on basal medium supplemented with kinetin and coconut milk.

1966- Torrey, J.G advanced the hypothesis that organogenesis in callus is initiated with the formation of clusters of meristematic cells called meristemoids.

1974- Murashige, T developed the concept of developmental stages in cultures *in vitro*: Stage I: Explant establishment; Stage II: Multiplication of propagule and Stage III: Rooting and hardening for planting into soil.

1981- Larkin, P.J. and Scowcroft, W.R developed the concept of **somaclonal variation**: A noval source of variability from cell cultures for plant improvement.

The history of plant tissue culture had its real beginning in 1934 when Gautheret tried to cultivate isolated cells and root tips on organised medium. The momentum from this pioneering work, a new turn in this ongoing research occurred, because of World War II. After the Second World War, American plant pathologists became interested in plant tissue culture.
As **Steward (1970)** pointed out, the plant tissue culture technique is another "**Silent Revolution in Agriculture**" having very good potentials to supplement conventional breeding approaches. Its potentials and prospects are discussed in subsequent chapters. The techniques' theoretical aspects and their applicabilities are simplified and presented.

Questions

1. The term totipotency was coined by ……..
   a) Duharmel du Monceau **b) Morgan, T.H**
   c) Haberlandt d) Rechinger

2. The term totipotency means ……..
a) the capability of a cell to form an individual plant **b)** the capability of a cell to form an individual cell
c) both a & b d) None of the above

1. Father of plant tissue culture is ……..
   a) Duharmel du Monceau b) Morgan, T.H
   c) Haberlandt d) Rechinger

2. The embryo culture was first demonstrated by ……..
   a) Robbins b) Harrison
   c) Liabach d) Kotte

3. Father of micropropagation is ……..
   a) Ball b) Harrison
   c) Robbins d) Kotte

4. The work on organogenesis in tobacco callus is first started by……..
   a) Ball **b) Skoog**
   c) Robbins d) White

5. ……. initiated studies on crown gall and tumour formation in plants
   a) White b) Braun
   c) Both a & b d) None of the above

6. Who demonstrated that virus free plants can be recovered from infected plants through shoot meristem culture
   a) Morel b) Martin
   c) Both a & b d) None of the above

7. The nurse culture technique was established by …….
   a) White b) Braun
   c) Muir d) None of the above

8. The first suspension culture by transferring callus fragments to agitated liquid medium was obtained by …….
   a) Hildebrandt b) Riker
   c) Muir **d) All the above**

9. Kinetin was identified by …….
   a) Miller b) Skoog
   c) Von Saltza and Strong **d) All the above**

10. The phenomenon of somatic embryogenesis in suspension culture of carrot was observed by …….
a) Steward b) Mapes
c) Mears **d) All the above**

11. The first suspension culture by transferring callus fragments to agitated liquid medium was obtained by …….
a) Hildebrandt b) Riker
c) Muir **d) All the above**

12. The somatic embryo formation from callus cultures of carrot grown on an agarified medium was observed by ……..
a) Hildebrandt **b) Reinert**
c) Muir d) All the above

13. the technique of isolation and culture of protoplasts after digesting the cell walls enzymatically was discovered by …….
a) Hildebrandt **b) Cocking**
c) Muir d) All the above

14. The plating technique was invented by …….
a) Bergmann b) Riker
c) Muir d) All the above

15. Meristem culture was discovered by …….
a) Bergmann b) Riker
c) Morel d) All the above

16. Anther culture was discovered by …….
a) Guha b) Maheshwari
c) Both a & b d) None of the above

17. The concept of developmental stages in cultures *in vitro* culture was developed by …….
a) Murashige b) Skoog
c) Both a & b d) None of the above

18. The concept of developmental stages in cultures *in vitro* culture was developed by …….
a) Murashige b) Skoog
c) Both a & b d) None of the above

Lecture 03 Tissue culture methodology: Sterile techniques

Establishing aseptic cultures

Plant tissue culture media contain sugar and so support the growth of many microorganisms (bacteria and fungi). When these microorganisms reach a medium, they generally grow much faster than the cultured plant materials. Their growth and toxic metabolites will affect, and may even kill, the tissue cultures. It is, therefore, essential to maintain a completely aseptic environment inside the culture vessels.

There are several possible sources of contamination of the medium:

- the culture vessel
- the medium itself
- the explant (plant tissue)
- the environment of the transfer area
- the instruments used to handle plant material during establishment and subculture
- the environment of the culture room.

Autoclaving media will eliminate contamination from the culture vessel or the medium. In some cases, substances such as gibberellic acid, abscisic acid (ABA), urea and certain vitamins are thermolabile and break down upon autoclaving. These chemicals can be sterilized by membrane filtration using microfilters of pore size 0.22-0.45 μm which is suitable enough to exclude pathogens. Later the filter sterilized compound can be added to autoclaved medium cooled to around 40°C.

To prevent the environment of the culture room from being the source of contamination, keep the culture room as dust- free as possible and remove contaminated cultures from the area as soon as they are detected. Ideally, the culture room should be clean, filtered air which has passed through high efficiency particulate air (HEPA) filters.

The transfer area in most laboratories is within a laminar air-flow cabinet. A laminar air-flow cabinet has a small fan which blows air through a coarse filter to remove large dust particles and then through a fine HEPA filter to remove microbes, their spores and other particles larger than 0.3 μm. The velocity of the air coming out of the fine filter is about 27 ± 3 m/min, which keeps airborne microorganisms out of the working area. The working area is swabbed with 70% alcohol (or equivalent) and instruments dipped in 70% alcohol, flamed and cooled before use.

Caution : Prolonged contact with alcohol can cause skin irritation, and other health problems can result from the inhalation of fumes. Use ethanol rather than methanol, and surgical gloves when handling. Take care with ultraviolet light as it can permanently damage eyes and promote skin cancer. Laminar flow cabinets equipped with ultraviolet light for surface sterilization should be fitted with safety doors which can be closed when ultraviolet light is used.

Plant surfaces carry a wide range of microorganisms. The tissue must be thoroughly surface-sterilized before being placed on the nutrient medium. Discard cultures with fungal or bacterial contamination. Solutions of sodium or calcium hypochlorite are usually effective in disinfecting plant tissues. Placing tissues in a 0.5 to 1% solution of sodium hypochlorite for 10 to 15 minutes will disinfect most tissues. Surface sterilants are toxic to plant tissues. Choose the concentration of the sterilizing agent and the length of time to minimize tissue damage, which shows up as white, bleached areas. Other techniques for surface sterilisation include dipping plant material for a few seconds in 90% ethanol or placing in running water for 30 minutes and 2 hours before disinfection.

Caution : Take care with powdered calcium hypochlorite as it is a powerful reducing agent. If calcium hypochlorite is stored moist and the container opened later, it can explode. Store calcium hypochlorite in a sealed container in a dry place.

A summary of the six steps commonly involved in establishing and maintaining aseptic plant tissue culture follows.

i. Collect pieces of plant material (ex-plants) in a screw-cap bottle. Immerse them in a dilute solution of the disinfectant containing a wetting agent. Replace the lid and store the bottle in the laminar air flow cabinet. Shake the bottle two or three times during the sterilization period.

ii. Remove the lid and drain carefully. Thoroughly rinse the plant material in sterilized distilled water and replace the lid. After shaking a few minutes, discard the water. Rinse two or three times more.

iii. Transfer the material to a pre-sterilized Petri-dishes or test-tubes.

iv. Sterilize the required instruments by dipping them in 70% ethanol and flamed them. Allow to cool. Sterilize the instruments after each time they are used to handle tissue.

v. Prepare suitable explants from the surface sterilized material using sterilized instruments (scalpels, needles, forceps, etc.).

vi. Quickly remove the lid of the culture vessel, transfer the explants on to the medium, flame the neck of the vessel (only if glass) and replace the lid.

vii. If handling aseptic plant materials during routine subculture, omit the first two steps.

Lecture 04
Totipotency and Morphogenesis

Totipotency

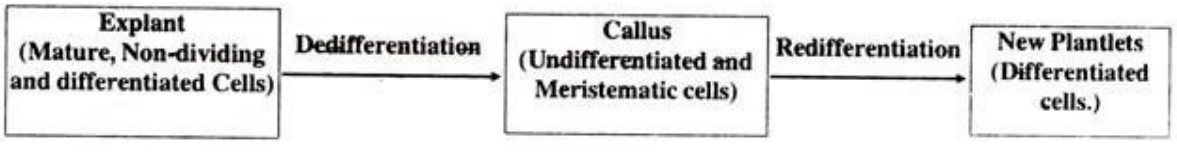
The inherent potentiality of a plant cell to give rise to a whole plant is described as cellular totipotency. This is a capacity which is retained even after a cell has undergone final differentiation in the plant body. In plants, even highly mature and differentiated cells retain the ability to regenerate to a meristematic state as long as they have an intact membrane system and a viable nucleus. This is contradicting to animals, where differentiation is generally irreversible.
For a differentiated cell, to express its totipotency, it first undergoes dedifferentiation followed by redifferentiation. **The phenomenon of a mature cell reverting to the meristematic state and forming undifferentiated callus tissue is termed 'dedifferentiation'. The phenomenon of conversion of component cells of callus tissue to whole plant or plant organs is called as 'redifferentiation'.**

Differentiation
The term differentiation is used in many different senses in biology. In broad sense, it is defined as the process by which meristematic cells are converted into two or more types of cells, tissues or organs which are qualitatively different from each other.

De-differentiation
The term is used to denote the process of formation of unorganised tissues from the highly organized tissues.

Re-differentiation
The process of differentiation occurring in an undifferentiated tissue.

Regeneration
It is defined as the structuring of any part, which has been removed or physiologically isolated from the organism. In other words, genesis of an entire plant from cultured explants directly or *via* callus indirectly is called regeneration.

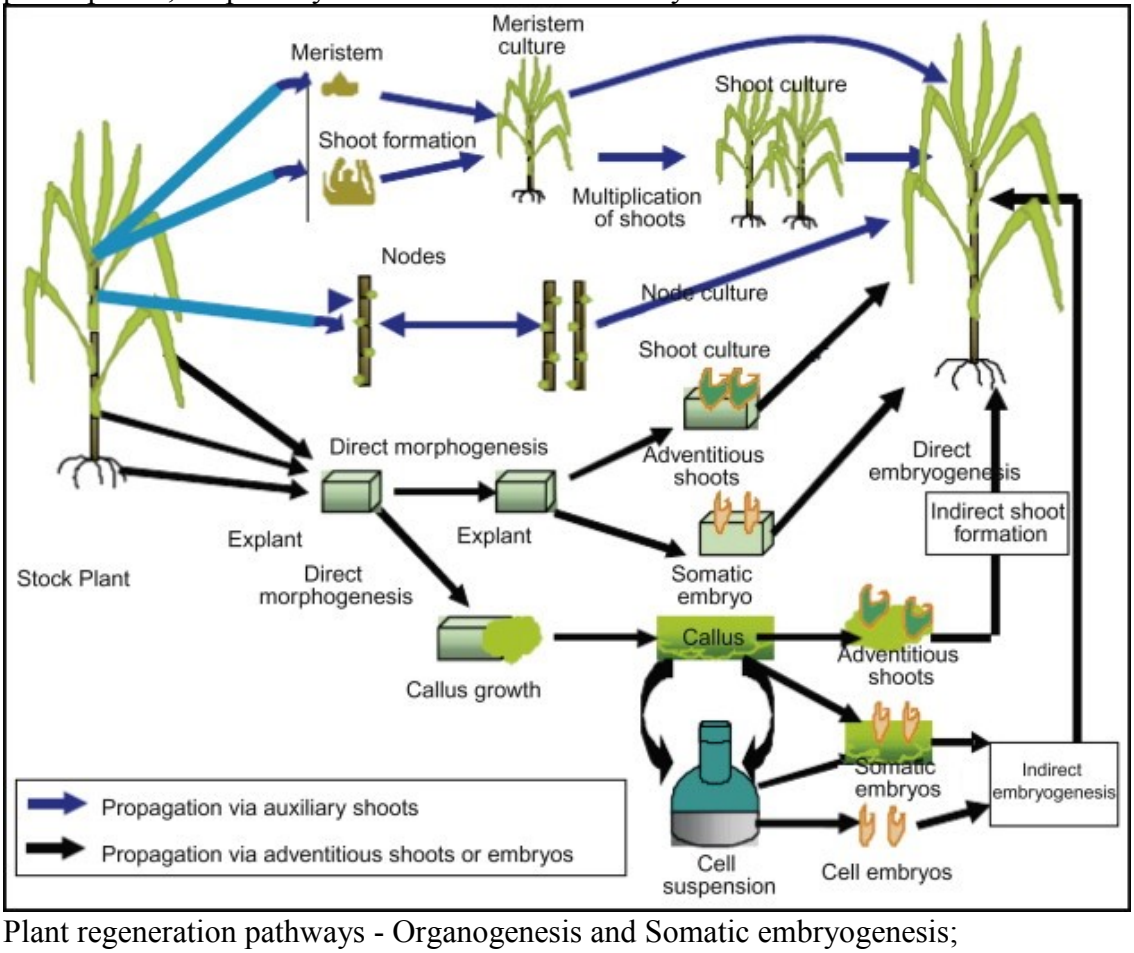


Totipotency, dedifferentiation; genetic control of regeneration;
What is the Meaning of Totipotency?
Totipotency is the genetic potential of a plant cell to produce the entire plant. In other words, totipotency is the cell characteristic in which the potential for forming all the cell types in the adult organism is retained.
Expression of Totipotency in Culture:
The basis of tissue culture is to grow large number of cells in a sterile controlled environment. The cells are obtained from stem, root or other plant parts and are allowed to grow in culture medium containing mineral nutrients, vitamins and hormones to encourage cell division and growth. As a result, the cells in culture will produce an unorganised proliferative mass of cells which is known as callus tissue.
Importance of Totipotency in Plant Science:

The ultimate objective in plant protoplast, cell and tissue culture is the reconstruction of plants from the totipotent cell. Although the process of differentiation is still mysterious in general, the expression of totipotent cell in cul-ture has provided a lot of information's. On the other hand, the totipotentiality of somatic cells has been exploited in vegetative propagation of many economical, medicinal as well as agricul-turally important plant species. Therefore, from fundamental to applied aspect of plant biology, cellular totipotency is highly important.

Recent trends of plant tissue culture include genetic modification of plants, production of homozy-gous diploid plants through haploid cell culture, somatic hybridization, mutation etc. The suc-cess of all these studies depends upon the expres-sion of totipotency. In many cases, successful and exciting results have been obtained.

Plant breeders, horticulturists and commercial plant growers are now more interested in plant tis-sue culture only for the exploitation of totipo-tent cells in culture according to their desirable requirement. Totipotent cells within a bit of cal-lus tissue can be stored in liquid nitrogen for a long period. Therefore, for germplasm preser-vation of endangered plant species, totipotency can be utilized successfully.



Plant regeneration pathways - Organogenesis and Somatic embryogenesis;
Organogenesis- Purpose, methods and requirements for organogenesis, indirect and direct organogenesis;
Somatic embryogenesis- Procedures and requirements for organogenesis, indirect and direct embryogenesis; Differences between somatic and gametic embryogenesis,

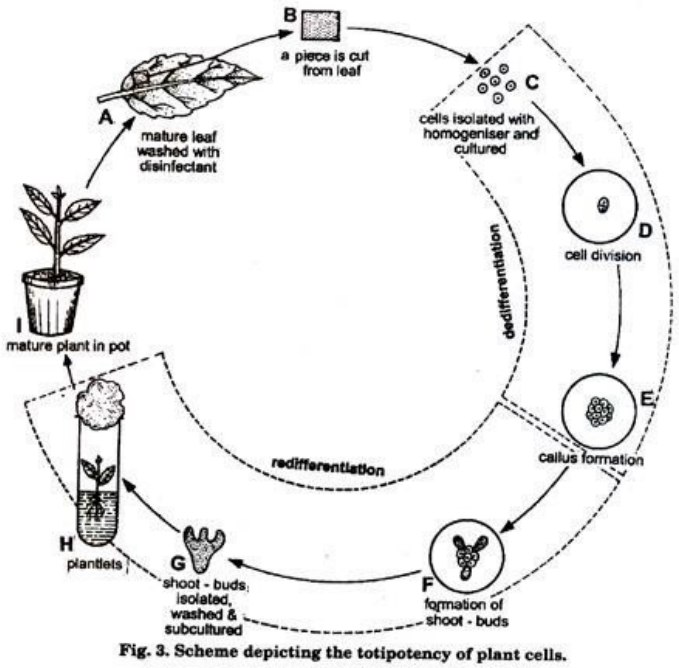


Fig. 3. Scheme depicting the totipotency of plant cells.

Morphogenesis

Attainment of biological organization or form is termed as morphogenesis. Under *in vitro* conditions this can be achieved by two routes: *de novo* origin of organs, either shoots or roots from the cultured tissues precisely termed as **organogenesis** and *de novo* origin of embryos with distinct root and shoot poles on opposite ends from the somatic cells or cells cultured *in vitro*, otherwise called as somatic embryogenesis

Figure 1. Stages of callus induction

The historical background, achievements and the causes for the two routes are discussed below.

Organogenesis

In plant tissue culture, organogenesis means genesis of organs like shoots, roots, leaves, flowers, *etc*. The earliest report on induction of shoot organogenesis *in vitro* was by White (1939) using a tobacco hybrid; and the first observation of root formation was reported by Nobecourt (1939) using carrot callus. Till late 1950s, the basic regulatory

mechanism underlying in organogenesis was not identified. Skoog and Miller (1957) were responsible to recognize the regulatory mechanism as a balance between auxin and cytokinin. As per their finding, a relatively high level of auxin to cytokinin favoured root formation and the reverse favoured shoot formation. Using this concept, it has now become possible to achieve organogenesis in a large number of plant species by culturing explants, calli and cell suspension in a defined medium.

In organogenesis, the shoot or root may form first depending upon the nature of growth hormones in the basal medium. The genesis of shoot and root from the explants or calli is termed as caulogenesis (caulm = stem) and rhizogenesis (rhizo = root) respectively.

Figure 2. Pathways of *in vitro* organogenesis

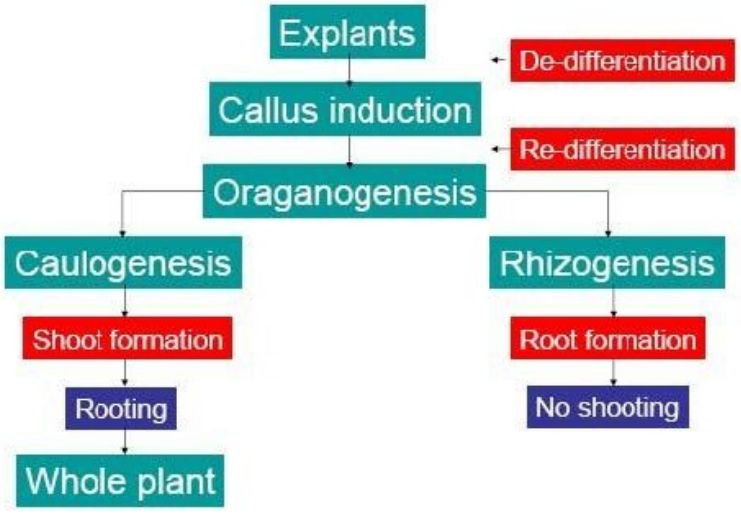
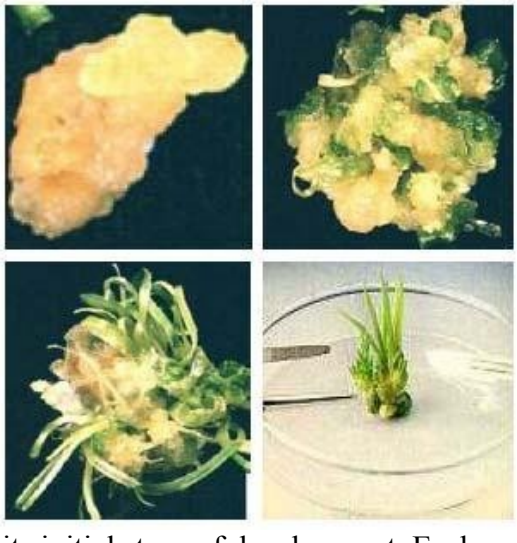


Figure 3. Stages of organogenesis

Embryogenesis

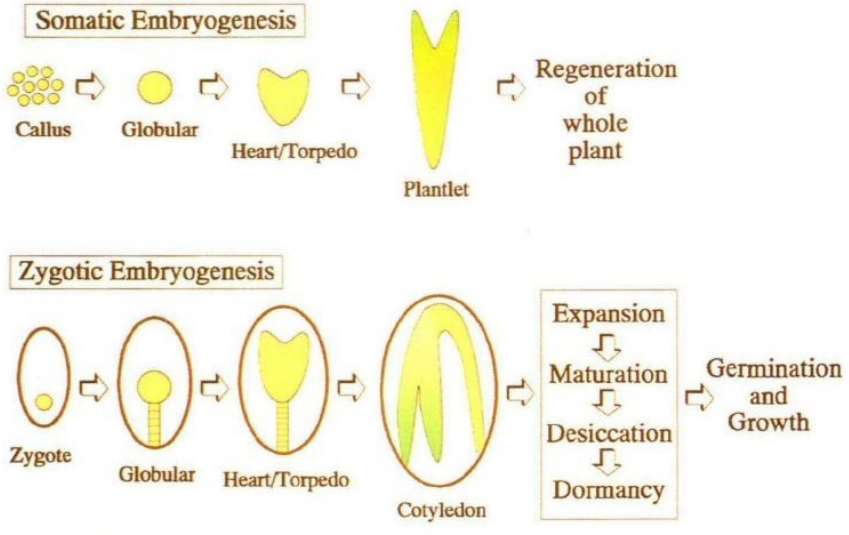


An embryo is defined as a plant in its initial stage of development. Each embryo possesses two distinct poles, one to form root and the other shoot, and is the product of fusion of gametes. In some plant species, embryos are produced without the fusion of

gametes and termed as asexual embryogenesis or adventitious embyrony.

In an intact plant this type of embryogenesis may occur in sporophytic tissues like integuments and nucellar tissues or from unfertilized gametic cells. Apart from the normal course of embryo formations *viz*., zygotic embryogenesis and adventitious embryony, instances of embryo formations from the tissues cultures *in vitro* were reported. This phenomenon termed as somatic embryogenesis was first observed by Steward and his co- workers (1958) in suspension cultures of carrot followed by Reinert (1959). Since then, a number of reports of embryo formation have been published.

Figure 4. A comparison of somatic and zygotic embryogenesis



Morphologically and developmentally, somatic and zygotic embryos are most similar from the globular stage through the torpedo stage. Somatic embryos do not experience desiccation or dormancy, but rather continue to grow into fully differentiated plantlets.

Somatic embryogenesis or embryogenesis *in vitro* produces embryo like structures resembling the zygotic embryos in structure and morphogenetic potential. Despite this resemblance, the ontogeny of an embryo like structure from somatic cell differs from that of zygotic embryo, where the origin is from a single cell.

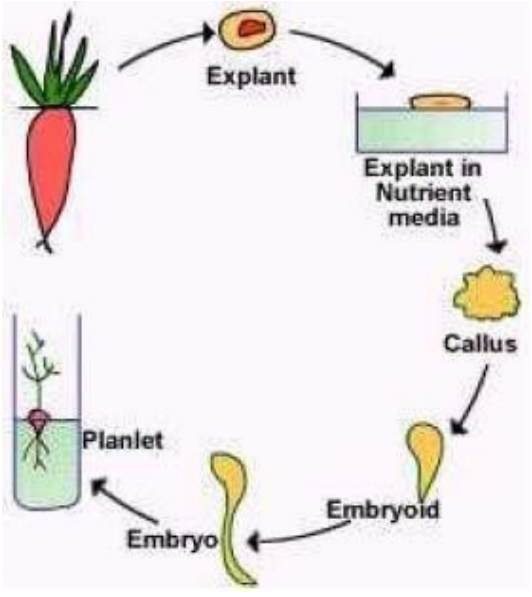


Figure 5. Pathway of somatic embryogenesis

Embryoid is generally used to denote the embryo like structure from cultured tissues. These embryoids possess bipolarity, no vascular connection with the mother tissue and origin from a single cell or a group of cells.

Theories on embryogenesis

Several theories have been proposed to explain the phenomenon of somatic embryogenesis, of which the following are considered important.

Cell isolation theory: Steward and his co-workers proposed this theory in 1964. According to them, the embryo producing cells are isolated from the neighbouring cells in a cell mass. The isolation of cells, favours the embryogenesis. The isolation of cell may be induced by the constraints in the surrounding cells, due to physical and physiological separation of cells. In most cases, the connection of plasmodesmata was severed. But this generally appears to be secondary to the induction process.

Differentiation theory: This theory states that the embryos would not be produced from the differentiated cells of the explants. The cells of explants have to undergo de-differentiation to form callus. Then the cells of callus will produce embryos. In other words, de- differentiation in cells is a prerequisite for the production of somatic embryos *in vitro*.

That the embryos can be formed directly from the epidermal cells of the stem or hypocotyl indicate the possibility of embryo formation without de-differentiation. The need for differentiation depends on the explant material used during primary culture. Epidermal cells of the stem, hypocotyl and young embryos may begin embryo development without going through a callus stage, while cortical cells and cells of xylem and phloem explants require de-differentiation. This theory was proposed by Halperin in 1970.

Intercellular communication and cytodifferentiation theory: According to this theory, cytodifferentiation in cells due to intercellular communication induces embryo formation. The cytodifferentiation is regulated by diffusion gradients of nutrients, endogenous plant growth regulators and gaseous factors like O2, CO2 and ethylene. The

changing microclimate in the culture environment affects intercellular communication and in turn cytodifferentiation. This concept was proposed by Street (1973).

Explant physiology and culture environment theory: This concept was developed by Street in 1976. He is of the view that the embryogenesis is a dependent phenomenon on the explant and the culture environment. Explants like flower buds, young embryos and parts of young seedling are most responsive to produce somatice embryos, but not from those of mature plants. Apart from the explant physiology, culture environment is also a factor influencing the embryogenesis. For example, highly embryogenic callus culture can be maintained non-embryogenic if the medium is supplemented with high level of auxin and the same may be induced to produce embryos when transferred to auxin free medium.

Pre-determination theory: This was proposed by Tisserat *et al*. (1979). It states that the embryo production potential is pre-determined phenomenon in the cells and the *in vitro* culture provides the opportunity for embryogenesis. In other words, embryogenesis from a cell is an inherent one which is facilitated to produce embryos by optimal culture environment.

Pre and induced embryogenic determined cell theory: Though the embryogenesis is pre-determined one there are instances of non-formation of embryos directly from the explants. In these cases, an intervening callus stage comes between the primary explant and the embryos. The cells in the calli are induced to produce embryos by the manipulation of medium with relevant growth regulator. Based on this, the above theory was proposed by Sharp and his co-workers. According to this theory, there are two types of embryogenic cells: pre-embryogenic determined cells (PEDC) and induced embryogenic determined cells (IEDC).

In pre-embryogenic determined embryogenic cells, embryogeny is determined prior to mitosis while induced embryogenic determined cells the embryogeny is induced by providing suitable mitogenic substance i.e., the embryogeny is induced in the cells of callus by the application of plant growth regulators. Thus in the callus, embryogenic precursor cells or embryogenic mother cells are formed which then develop into embryogenic cells. Later these cells undergo polarised cell divisions typical of normal embryogenesis by forming globular, heart and torpedo shaped embryos.

Events during embryogenesis

In 1959, Reinert made the remarkable claim that following a succession of changes of the nutrient media, root derived callus tissue of *Daucas carota* produced normal bipolar embryos. The changes made or observed in the nutrient medium were as follows: maintenance of callus in White's medium with high level of auxin (IAA at 10 mg/litre) and subculturing of callus for several months on White's basal medium with additives like
vitamins, amino acids, amides and purines. As a result of these manipulations, the calli showed small protuberances on the surface. Histological sections of these calli showed centres of organised development. These tissues with organised centres, on transfer to auxin lacking but coconut milk containing medium produced embryoids and from embryoids, whole plants.

Figure 6. Stages of somatic embryogenesis

Patterns of embryogenesis

Two general patterns of embryogenesis *in vitro* are identified. Origin of embryos directly from the tissue cultured *in vitro* (direct embryogenesis) and origin of embryos *via* callus stage (indirect embryogenesis).

Differences between direct and indirect embryogenesis

Direct embryogenesis	**Indirect embryogenesis**
Embryos form from the explants directly	Embryos arise from the callus induced from the explants
A promoting substance to induce the embryo formation is needed	Auxin is need to induce callus, and cytokinin is needed to induce differentiation
The embryogenic nature of a cell is predetermined	The embryogenic nature of a cell is induced in the culture
The origin of embryos mostly from individual cells; sometimes from a group of cells	The origin may be either from single cells or from a group of cells called pro- embryonal complex

Questions

1. Cell differentiation in animals is ……..
   a) Irreversible b) Reversible
   c) Both a & b d) None of the above
2. Cell differentiation in plants is ……..
   a) Irreversible **b) Reversible**
   c) Both a & b d) None of tha above
3. The inherent potentiality of a plant cell to give rise to a whole plant is described as ……..
   a) Cellular totipotency **b) Morphogenesis**
   c) Organogenesisd) None of the above
4. For a differentiated cell, to express its totipotency, it should undergo dedifferentiation followed by redifferentiation
   a) Dedifferentiation b) Redifferentiation
   c) Both a & b d) None of the above
5. The phenomenon of a mature cell reverting to the meristematic state and forming undifferentiated callus tissue is termed as ……..
   a) Dedifferentiation b) Redifferentiation
   c) Both a & b d) None of the above

6. The phenomenon of conversion of component cells of callus tissue to whole plant or plant organs is called as ……..
   a) Dedifferentiation **b) Redifferentiation**
   c) Both a & b d) None of the above

7. The phenomenon of totipotency is demonstrated with the experiment involving ……..
   a) Beetroot b) Onion
   c) Carrotd) None of the above

8. The phenomenon of morphogenesis depends on the factors like……..
   a) Polarity b) Differentiation
   c) Regeneration of individual cells **d) All the above**

9. …….. concluded in "Organbildung im Pflanzenseichconcluded that the fate of a cell is a function of its position.
   a) Vochting b) Haberlandt
   c) Hanstein d) Klebs

10. Under *in vitro* conditions this can be achieved by …….. .
    a) *de novo* origin of organs b) *de novo* origin of embryos
    c) Both a & b d) None of the above
11. Organogenesis means genesis of organ(s) like …….. .
    a) shoots b) roots
    c) leaves and flowers **d) All the above**
12. The earliest report on induction of shoot organogenesis *in vitro* was by …….. using a tobacco hybrid.
    a) White b) Nobecourt
    c) Skoog d) Miller
13. The first observation of root formation was reported by …….. using carrot callus.
    a) White **b) Nobecourt**
    c) Skoog d) Miller

14. The regulatory mechanism ie., balance between auxin and cytokinin is recognized by……..
a) White **b) Nobecourt**
c) Skoog & Miller d) Murashigee

15. The relatively high level of auxin to cytokinin favoured …….. during organogenesis
a) Root formation b) Shoot formation
c) Both root and shoot formation d) None of the above

16. The relatively high level of cytokinin to auxin favoured …….. during organogenesis.
a) Root formation **b) Shoot formation**
c) Both root and shoot formation d) None of the above

17. The genesis of shoot from the explants or calli is termed as ……..
a) Caulogenesis b) Rhizogenesis
c) Organogenesisd) None of the above

18. The genesis of root from the explants or calli is termed as ……..
a) Caulogenesis **b) Rhizogenesis**
c) Organogenesisd) None of the above

19. The event of organogenesis is discovered by ……..
a) Torreyb) Skoog
c) Guha d) None of the above

20. The somatic embryogenesis was first observed by ……..
a) Steward b) Skoog
c) Reinertd) None of the above

21. High concentration of …… favours shoot initiation.
a) IAA **b) Kinetin**
c) GA_3 d) None of the above

22. High concentration of …… favours rooting.
a) Auxin b) Cytokinin
c) Gibberellic acid d) None of the above

23. In somatic embryogenesis …… is required for induction of embryonic cells and maintenance of proliferative growth..
a) Auxin b) Cytokinin
c) Gibberellic acid d) None of the above

24. Embryo formation can be induced by transferring the callus to a medium lacking …….
a) Auxin b) Cytokinin
c) Gibberellic acid d) None of the above

25. …… is used most successfully to obtain rapid growth of somatic embryos into plants.
a) Auxin b) Cytokinin
c) Gibberellic acid d) None of the above

Lecture 05
Synthetic and natural media components, growth regulators, environmental requirement.
Types of media – Solid and liquid media – Advantages and limitations

Culture medium is a general term used for the liquid (or) solidified formulations upon which plant cells, tissues (or) organs develop in the plant tissue culture. Thus normally the explants are grown in two different types of media

Solid Medium

Liquid Medium

Solid Medium:-

A solidifying or a gelling agent is commonly used for preparing semisolid (or) solid tissue culture medium. The plant material is placed on the surface of the medium. The tissue remains intact and the cell multiplication is comparatively slow.

Advantages

solid medium is most widely used in plant tissue culture because of its simplicity and easy handling nature. 2) Acquires sufficient aeration without a special device since the plant material is placed on the surface of the medium.

Disadvantages

Only a part of the explant is incontact with the surface of the medium. Hence there may be inequality in growth response of tissues and there may be a nutrient gradient between callus and medium

Culture media are largely responsible for the in vitro growth and morphogenesis of plant tissues. The success of the plant tissue culture depends on the choice of the nutrient medium. In fact, the cells of most plant cells can be grown in culture media.
Synthetic and natural media:
When a medium is composed of chemically defined components, it is referred to as a synthetic medium. On the other hand, if a medium contains chemically undefined compounds (e.g., vegetable extract, fruit juice, plant extract), it is regarded as a natural medium. Synthetic media have almost replaced the natural media for tissue culture.

Composition of widely used tissue culture media
i. **Macronutrients:** As its name suggests, in plant tissue culture media these components provide the elements which are required in large amounts (concentrations greater than 0.5 mmole l^{-1}) by cultured plant cells. Macronutrients are usually considered to be carbon, nitrogen, phosphorous, magnesium, potassium, calcium and sulphur.
ii. **Micronutrients:** It provides the elements that are required in trace amounts (concentrations less than 0.5 mmole l^{-1}) for plant growth and development. These include, manganese, copper, cobalt, boron, iron, molybdenum, zinc and iodine.
iii. **Iron source**: It is considered the most important constituent and required for the formation of several chlorophyll precursors and is a component of ferredoxins (proteins containing iron) which are important oxidation : reduction reagents.

iv. Organic supplements (vitamins): Like animals, in plants too vitamins provide nutrition for healthy growth and development. Although plants synthesize many vitamins under natural conditions and, therefore, under in vitro conditions they are supplied from outside to maintain biosynthetic capacity of plant cells in vitro. There are no firm rules as to what vitamins are essential for plant tissues and cell cultures. The only two vitamins that are considered to be essential are myo-inositol and thiamine. Myo-inositol is considered to be vitamin B and has many diverse roles in cellular metabolism and physiology. It is also involved in the biosynthesis of vitamin C.

v. Carbon source: This is supplied in the form of carbohydrate. Plant cells and tissues in the culture medium are heterotrophic and are dependent on external source of carbon. Sucrose is the preferred carbon source as it is economical, readily available, relatively stable to autoclaving and readily assimilated by plant cells. During sterilization (by autoclaving) of medium, sucrose gets hydrolyzed to glucose and fructose. Plant cells in culture first utilize glucose and then fructose. Besides sucrose, other carbohydrates such as, lactose, maltose, galactose are also used in culture media but with a very limited success.

Dilutions:

$$\frac{\text{required concentration} \times \text{medium volume}}{\text{concentration of stock solution}} = \text{volume of stock required}$$

Gelling agents

The media listed above are only for liquids, often in plant cell culture a 'semi-solid' medium is used. To make a semi-solid medium, a gelling agent is added to the liquid medium before autoclaving. Gelling agents are usually polymers that set on cooling after autoclaving.

i. Agar: Agar is obtained from red algae- *Gelidium amansii* . It is a mixture of polysaccharides. It is used as a gelling agent due to the reasons: (a) It does not react with the media constituents (b) It is not digested by plant enzymes and is stable at culture temperature.

ii. Agarose: It is obtained by purifying agar to remove the agaropectins. This is required where high gel strength is needed, such as in single cell or protoplast cultures.

iii. Gelrite: It is produced by bacterium *Pseudomonas elodea* . It can be readily prepared in cold solution at room temperature. It sets as a clear gel which assists easy observation of cultures and their possible contamination. Unlike agar, the gel strength of gelrite is unaffected over a wide range of pH. However, few plants show hyperhydricity on gelrite due to freely available water.

iv. Gelatin: It is used at a high concentration (10%) with a limited success. This is mainly because gelatin melts at low temperature (25°C) and as a result the gelling property is lost.

Phytohormones

These are organic compounds, other than nutrients, which influence growth, differentiation and multiplication. They required in very minute quantity in the media. The requirement for these substances varies considerably with the tissue and it also depends on their endogenous level. There are many commercially available synthetic substances that mimics the PGR specific to certain species. Testing of various types, concentrations and mixtures of growth substances during the development of a tissue culture protocol for a new species is essential before using a new PGR in plant tissue culture.

There are different groups of PGRs commonly used in the media. They are auxins, cytokinins, gibberellins, ethylene and absicissic acid. Additional substances gaining recognition as hormones in plant tissue culture are: polyamines, jasmonates, salicylic acid and brassinosteroids.

1. Auxin

In nature, the hormones of this group are involved with elongation of stem, internodes, tropism, apical dominance, abscission, rooting etc. In tissue culture auxins have been used for cell division and root differentiation. The commonly used auxins in tissue culture are

1. Indole-3-acetic acid (IAA)
2. Indole-3-butyric acid (IBA)
3. Naphthalene acetic acid (NAA)
4. Dichlorophenoxyacetic acid (2, 4-D)

Auxins are usually dissolved in either ethanol or dilute NaOH.

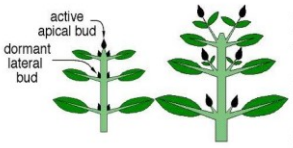
Effects of different Auxin on Plant Growth and Development

➢ **Apical Dominance**

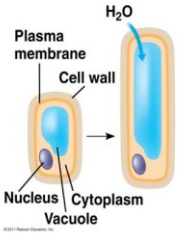
- Apical or terminal buds of many vascular plants are very active while the lateral buds remain inactive.
- Removal of apical buds promotes lateral buds to grow.
- Apical dominance is due to much higher auxin content in the apical buds than lateral buds.

➢ **Phototropism**

- Plant bend towards unilateral light.
- This is due to higher concentration of auxin on the shaded side.



Pinching of apical bud induces growth of lateral buds under influence of cytokinins



Auxin induces cell elongation thru uptake of water in vacuole and loosening of cell wall

2. Cytokinins

These hormones are essential for cell division, modification of apical dominance, shoot differentiation etc. In tissue culture media, cytokinins are incorporated mainly for cell division, differentiation of adventitious shoots from callus and organ & shoot proliferation. Commonly used cytokinins are

1. Benzylamino purine (BAP)
2. Isopentenyl adenine (2-ip)
3. Furfurylamino purine (kinetin)
4. Zeatin

Cytokinins are generally dissolved in dilute HCl or NaOH.

Auxin - Cytokinin Interaction

1. High auxin and low cytokinin ratio: Initialize root formation, embryogenesis and callus formation.
2. Low auxin and high cytokinin ratio: Induce formation of adventitive or axillary shoots.
3. The auxin-cytokinin ratio is also essential for chloroplast formation and other processes.

Effect of different auxin and cytokinin concentration on tissue development

3. Gibberellins

Naturally occurring plant hormones involved in internode elongation, enhancement of flower, fruit and leaf size, germination and vernalization in plants. Among the 20 known gibberellins, GA_3 is used widely. Compared to auxins and cytokinins, gibberellins are used very rarely. They stimulate normal development of plantlets from *in vitro* formed adventitious embryos. They are soluble in cold water.

4. Ethylene

A gaseous plant hormone involved in fruit maturation, abscission, and senescence. All kinds of plant tissue cultures produce ethylene and the rate of production increases under stress conditions. Use of ethylene precursor (2-chloroethylphosphonic acid) in tissue culture may be promotory or inhibitory for the same process in different species. For example, it promoted somatic embryogenesis in *Zea mays* whereas the same process was inhibited in *Hevea brasiliensis.*

5. Abscisic acid

A plant hormone involved in abscission, enforcing dormancy and regulating early stages of embryo development. It is required for normal growth and development of somatic embryos and promotes morphogenesis.

6. Brassinosteroids

It promotes shoot elongation at low concentrations and strongly inhibits root growth and development. It also promotes ethylene biosynthesis and epinasty.

7. Jasmonates

Jasmonates are represented by jasmonic acid and it is a methyl ester. Jasmonic acid is considered to be a new class of plant growth substance. It inhibits many processes such as embryogenesis, seed germination, pollen germination, flower bud formation, chlorophyll formation. It is involved in differentiation, adventitious root formation, breaking of seed dormancy and pollen germination.

8. Polyamines

There is some controversy as to whether these compounds should be classified with hormones. They appear to be essential in growth and cell division.

9. Salicylic acid

It is thought to be a new class of plant growth substances**.** It promotes flowering, inhibits ethylene biosynthesis and reverses the effects of ABA.

Environmental factors influencing plant tissue culture

1. Genotype or variety of the plant material
2. Explant selection and its size.
3. Medium:

(i) Nutrients,
(ii) Growth regulators and
(iii) Other additives

4. Culture Environment:

(i) Temperature,
(ii) Relative Humidity (RH) and
(iii) Light

Hydrogen ion concentration (pH)

The pH of the medium is usually adjusted between 5.0 and 6.0 prior to the addition of agar and autoclaving. The extremes of pH should be avoided as this will block the availability of some of nutrients to the inoculum. A pH of 5.8 is found to be optimum for plant tissue culture. Generally, pH higher than 6.0 give a very hard medium and a pH below 5.0 do not allow satisfactory solidification of agar. Further, pH in media changes during growth of plant tissues and this drift in pH is comparatively low in media with high salt concentration, because of their greater buffering capacity.

Questions

1. The mineral solution widely used by early investigators was …….
   a) Knop's medium b) MS medium
   c) White's medium d) None of the above

2. The medium in tissue culture should provide …….
a) Nutrients for the survival of the plant cells, tissues and organs under culture
b) Optimal physical condition of pH, osmotic pressure, *etc*
c) **Both a & b** d) None of the above

3. The callus culture was first developed by …….
   a) Skoog b) White
   c) Gautherat d) None of the above

4. The systematic study of mineral requirements of plant tissue and organs in culture was made by
   a) Murashige and Skoog b) Vasil and Hildebrant
   c) Nitsch and Nitsch **d) All the above**

5. The mineral nutrient that plays a vital role in growth and differentiation of cultured tissues is …
   a) N b) P
   c) K d) All the above

6. Nitrogen is generally supplied in ……. form.
   a) NH4 b) NO3
   c) NO2 d) All the above

7. The range of inorganic nitrogen varies between …….. in the tissue culture medium.
   a) 25 mM - 60 mM b) 250 mM - 600 mM
   c) 2.5 mM – 6.0 mM d) 0.25 mM – 0.60 mM

8. Phosphorus levels greater than ……. are inhibitory to growth of tissues.
   a) 2 mM b) 20 mM
   c) 200 mM d) 0.2 mM

9. The optimum concentration of K needed is …….
   a) 2 mM **b) 20 mM**
   c) 200 mM d) 0.2 mM

10. At low nitrogen concentration presence of …….. element enhances the formation of somatic embryos
    a) K b) P
    c)Ca d) Mg

11. Sulphur is provided in the form of sulphates
    a) Sulphates b) Sulphites
    c) Both a & b d) None of the above

12. The standard carbon source is ……..
    a) Sucrose (2-5 per cent) b) Glucose
    c) Fructose d) None of the above

13. The standard carbon source is ……..
a) Sucrose (2-5 per cent) b) Glucose
c) Fructose d) None of the above

14. Universally required vitamin in tissue culture medium is ……..
a) Thiamine HCL b) Pyridoxine HCL
c) Nicotinic acid d) Calcium pantothanate

15. Vitamin (s) used in tissue culture medium is/are ……..
a) Calcium pantothanate b) Pyridoxine HCL
c) Nicotinic acid **d) All the above**

16. The commonly used auxins in tissue culture are ……..
a) IAA, IBA, NAA, 2,4 D b) BAP, 2-ip, Kinetin, Zeatin
c) GA_3 d) All the above

17. The commonly used cytokinins in tissue culture are ……..
a) IAA, IBA, NAA, 2,4 D **b) BAP, 2-ip, Kinetin, Zeatin**
c) GA_3 d) All the above

18. The auxins are diluted in ……..
a) Ethanol b) Dilute NaOH
c) Both a & b d) None of the above

19. The cytokinins are diluted in ……..
a) Dilute HCl b) Dilute NaOH
c) Both a & b d) None of the above

20. In tissue culture auxins have been used for …….
a) Cell division and root differentiation b) Differentiation of adventitious shoots from callus and organ & shoot proliferation
c) Normal development of plantlets from *in vitro* formed adventitious embryos
d) None of the above

21. In tissue culture cytokinins have been used for …….
a) Cell division and root differentiation **b) Differentiation of adventitious shoots from callus and organ & shoot proliferation**
c) Normal development of plantlets from*in vitro* formed adventitious embryos
d) None of the above

22. In tissue culture gibberellins have been used for …….
a) Cell division and root differentiation b) Differentiation of adventitious shoots from callus and organ & shoot proliferation
c) Normal development of plantlets from *in vitro* formed adventitious embryos
d) None of the above

23. High auxin and low cytokinin ratio favours …….
a) Initialize root formation, embryogenesis and callus formation **b)** Induce formation of adventitive or axillary shoots
c) Chloroplast formation and other processes d) None of the above

24. Low auxin and high cytokinin ratio favours …….

a) Initialize root formation, embryogenesis and callus formation
b) Induce formation of adventitive or axillary shoots
c) Chloroplast formation and other processes
d) None of the above

25. The auxin-cytokinin ratio is essential for …….

a) Initialize root formation, embryogenesis and callus formation
b) Induce formation ofadventitive or axillary shoots
c) Chloroplast formation and other processes
d) All the above

26. The naturally occurring gibberellin is involved in …….

a) Internode elongation b) Enhancement of flower, fruit and leaf size
c) Germination and vernalization **d) All the above**

27. The gibberellins are soluble in ……..

a) Dilute HCl b) Dilute NaOH
c) Cold water d) Hot water

28. Ethylene is involved in ……..

a) Fruit maturation b) Abscission
c) Senescence **d) All the above**

29. Abscissic acid is involved in ……..

a) Enforcing dormancy b) Abscission
c) Regulating early stages of embryo development **d) All the above**

30. Abscissic acid is required for ……..

a) Normal growthb) **P**romotes morphogenesis
c) Development of somatic embryos **d) All the above**

31. …….. promotes ethylene biosynthesis and epinasty.

a) Brassinosteroids b) Jasmonates
c) Polyamines d) Salicyclic acid

32. Jasmonic acid is a ……..

a) Methyl ester b) Ethyl ester
c) Ether d) None of the above

33. The optimum pH of the tissue culture is ……...

a) 5.8 b) 6.0
c) 4.0 d) 7.0

34. The substitutes for agar are

a) Gelatinb) Silica gel
c) Acryl amide gel **d) All the above**

35. The osmoticums used in tissue culture medium are

a) Sorbitol and mannitol b) Sucrose
c) Fructose and galactose **d) All the above**

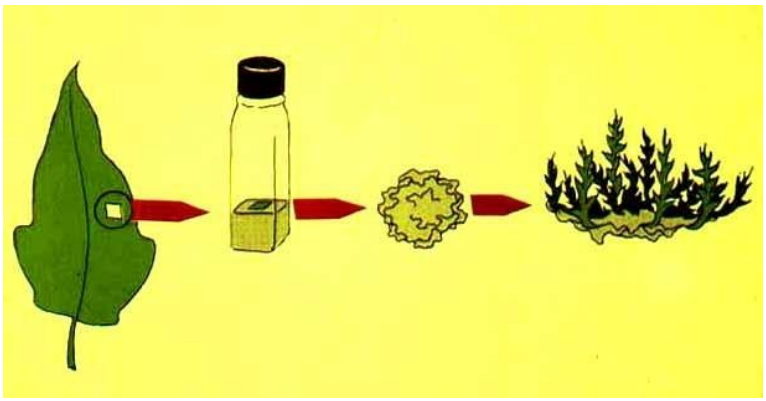
Lecture 06
Micropropagation

Multiplication of genetically identical copies of a cultivar by asexual reproduction is called *clonal propagation*. In nature, clonal propagation occurs by apomixis (seed development without meiosis and fertilization) and/or vegetative propagation (regeneration of new plants from vegetative parts). Tissue culture has become popular method for vegetative propagation of plants. Aseptic method of clonal propagation is called as *Micropropagation* and it offer the advantage of large number of true-to-type plantlets can be produced with relatively short time and space from a single individual. It is the fact that micropropagation is the only commercially viable method of clonal propagation of most of the horticultural crops. E.g. Orchids.

Explants used in micropropagation

Different kinds of explants were used in micropropagation. For example, in case of orchids, shoot tip (*Anacamptis pyramidalis, Aranthera, Calanthe, Dendrobium),* axillary bud (*Aranda, Brassocattleya, Cattleya, Laelia)*, inflorescence segment (*Aranda, Ascofinetia, Neostylis, Vascostylis)*, lateral bud (*Cattleya, Rhynocostylis gigantean)*, leaf base (*Cattleya)*, leaf tip (*Cattleya, Epidendrum),* shoot tip (*Cymbidium, Dendrobium, Odontioda, Odontonia),* nodal segment (*Dendrobium),* flower stalk segment (*Dendrobium, Phalaenopsis)* and root tips (*Neottia, Vanilla)* are being used in micropropagation.

Different explants



Differentiation of shoots directly from callus

Stages in micropropagation

Micropropagation generally involves five stages. Each stage has its own requirements.

Stage 0: Preparative stage

This stage involves the preparation of mother plants to provide quality explants for better establishment of aseptic cultures in stage 1. To reduce the contamination problem in the subsequent stages, mother plant should be grown in a glasshouse and watered so as to avoid overhead irrigation. This will also reduce the need for a harsh sterilization treatment. Stage 0 also includes exposing the stock plants to suitable light, temperature and growth regulator treatments to improve the quality of explants. In the case of photosensitive plants it may be possible to obtain suitable explants throughout the year by controlling photoperiod in the glasshouse. For example, red-light treated plants of *Petunia* provided leaf explants which produced up to three times as many shoots as did the explants from untreated plants.

Stage 1. Initiation of culture

1. **Explant:** The nature of explant to be used for *in vitro* propagation is governed by the method of

www.AgriMoon.Com

shoot multiplication. For enhanced axillary branching, only the explants which carry a pre-formed vegetative bud are suitable. When the objective is to produce virus-free plants from an infected individual it becomes necessary to start with sub-millimeter shoot tips. If the stock is virus-tested or virus eradication is not necessary, then the most suitable explant is nodal cuttings. Small shoot-tip explants have a low survival rate and show slow initial growth. Meristem tip culture may also result in the loss of certain horticultural characteristics which are controlled by the presence of virus, such as the clear-vein character of the Geranium cv. Crocodile. Generally, the clear vein character is transmitted in petiole-segment culture but not in shoot-tip culture.

2. **Sterilization:** Special precautions need to be taken when explants are derived from field- grown materials, which is often necessary in cloning an elite tree. In such cases an ideal approach would be to take cuttings from the selected plant and grow them in greenhouse. Discarding the surface tissues from plant materials while preparing the explants also minimizes the loss of cultures due to microbial contamination.

3. **Browning of medium:** A serious problem with the culture of some plant species is the oxidation of phenolic compounds leached out from the cut surface of the explant. It turns the medium dark brown and is often toxic to the tissues. This problem is common with the adult tissues from woody species.

3. Stage 2. Multiplication

This is the most crucial stage since it is the point at which most of failures in micropropagation occur. Broadly three approaches have been followed to achieve *in vitro* multiplication.

1. **Through callusing:** The potentiality of plant cells to multiply indefinitely in cultures and their totipotent nature permit a very rapid multiplication of several plant types. Differentiation of plants from cultured cells may occur via shoot-root formation (organogenesis) or somatic embryogenesis. Somatic embryogenesis is most appealing from a commercial angle. A somatic embryogenesis system once established lends itself to better control than organogenesis. Since somatic embryos are bipolar structures, with defined root and shoot meristems, the rooting stage required for microshoots gets eliminated. Above all, somatic embryos being small, uniform and bipolar are more amenable to automation at the multiplication stage and for field planting as synthetic seeds, offering cost advantages from labour savings, can also be stored through cold storage, cryopreservation or desiccation for prolonged periods. These characteristics make somatic embryogenesis potentially a less expensive and flexible system for micropropagation. The most serious objection against the use of callus cultures for shoot multiplication is the genetic instability of their cells.

2. **Adventitious bud formation:** Buds arising from any place other than leaf axil or the shoot apex are termed adventitious buds. The shoots differentiated from calli should also be treated as adventitious buds. In many crops, vegetative propagation through adventitious bud formation from root (blackberry, raspberry) and leaf (*Begonia, Crassula)* cuttings is standard horticultural practice. In such cases the rate of adventitious bud development can be considerably enhanced under culture conditions. For most bulbous plants (e.g. Lilley) adventitious bud formation is the most important mode of multiplication and the best
explants are obtained from bulb scales. A serious problem may arise when this method of propagation is applied to varieties which are genetic chimeras. Adventitious bud formation involves the risk of splitting the chimeras leading to pure type plants. For example, in variegated geranium cv. Mme Salleron, the chimera is perpetuated in meristem culture but broken down in petiole culture.

www.AgriMoon.Com

3. **Enhanced axillary branching:** In cultures the rate of shoot multiplication by enhanced axillary branching can be substantially enhanced by growing shoots in a medium containing a suitable cytokinin at an appropriate concentration with or without auxin. Due to continuous availability of cytokinin, the shoots formed by the bud, present on the explant, develops axillary buds which may grow directly into shoots. This process may be repeated several times and the initial explant transformed into a mass of branches.

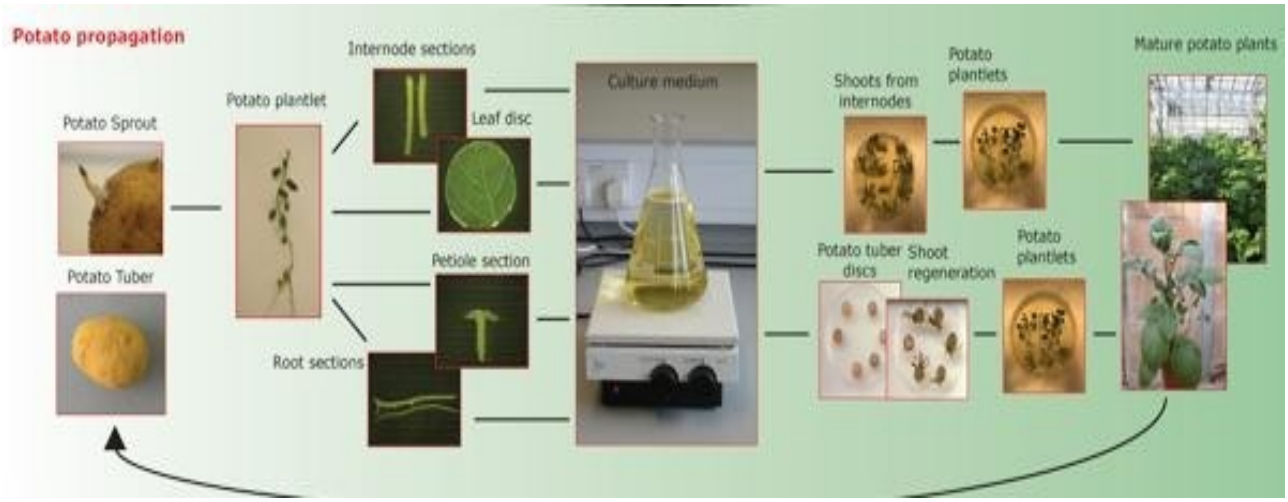
4. Stage 3. Rooting of shoots

Somatic embryos carry a pre-formed radical and may develop directly into plantlet. However, these embryos often show very poor conversion into plantlets, especially under *in vitro* conditions. They require an additional step of maturation to acquire the capability for normal germination. Adventitious and axillary shoots developed in cultures in the presence of a cytokinin generally lack roots. To obtain full plants the shoots must be transferred to a rooting medium which is different from the shoot multiplication medium, particularly in its hormonal and salt compositions. For rooting, individual shoots measuring 2 cm in length are excised and transferred to the rooting medium.

5. Stage 4. Transplantation

The ultimate success of commercial propagation depends on the ability to transfer plants out of culture on a large scale, at low cost and with high survival rates. The plants multiplied *in vitro* are exposed to a unique set of growth conditions (high levels of inorganic and organic nutrients, growth regulators, sucrose as carbon source, high humidity, low light, poor gaseous exchange) which may support rapid growth and multiplication but also induce structural and physiological abnormalities in the plants, rendering them unfit for survival under *in vivo* conditions. The two main deficiencies of *in vitro* grown plants are – poor control of water loss and heterotrophic mode of nutrition. Therefore, gradual acclimatization is necessary for these plants to survive transition from culture to the greenhouse or field. During acclimatization the *in vitro* formed leaves do not recover but the plant develops normal leaves and functional roots. While transferring out shoots/roots their lower part is gently washed to remove the medium sticking to them. The individual shoots or plantlets are then transferred to potting mix and irrigated with low concentration of inorganic nutrients. This probably recommissions the photosynthetic machinery of plants, enabling them to withstand the subsequent reduction in the ambient relative humidity and survive under field conditions. A variety of potting mixtures such as peat, perlite, polystyrene beads, vermiculate, fine bark, coarse sand etc. or their mixtures in different combinations are used for transplantation. For initial 10-15 days, it is essential to maintain high humidity (90-100%) around the plants, to which they got adapted during culture. The humidity is gradually reduced to ambient level over a period of 2-4 weeks.

www.AgriMoon.Com



Advantages of micropropagation

1. Clonal mass propagation - extremely large numbers of plants can be produced. Rather than getting 10000 plants per year from an initial cutting in vegetative propagation, one can obtain more than 1,000,000 plants per year from one initial explant through micropropagation.
2. Culture is initialized from small parts of plants – so no need of much space: from 1 m^2 space in culture room, 20000 - 100000 plants can be produced per year.
3. Production of disease and virus free plantlets. This leads to simplification of international exchange of plants
4. Micropropagation enables growers to increase the production of plants that normally propagate very slowly such as *Narcissus* and other bulbous crops.
5. Introduction of disease free new cultivars is possible through micropropagation
6. Vegetative propagation of sterile hybrids can be used as parent plants for seed production. Eg. Cabbage
7. One of the rapid methods for cloning of disease free trees.
8. *In vitro* cultures can be stored for long time through cryopreservation.
9. Breeding cycle can be shortened.

Disadvantages of micropropagation

1. Expensive laboratory equipment and service
2. No possibility of using mechanization
3. Plants are not autotrophic
4. Poor Acclimatization to the field is a common problem (hyperhydricity)
5. Risk of genetic changes if 'de novo' regeneration is used
6. Mass propagation cannot be done with all crops to date. In cereals much less success is achieved
7. Regeneration is often not possible, especially with adult woody plant material.
8. More problems in inducing rooting
9. May not get uniform growth of original plant from tissue culture. Each explant has different *in vitro* growth rates and maturation. Thus cannot be used for floriculture crop production where uniformity is critical.

Horticultural uses for plant tissue culture

1. **Clonal mass propagation**. The important point here is that extremely large numbers of plants can be produced. Rather than getting 10000 plants per year from an initial cutting, one can obtain upwards of 1,000,000 plants per year from one initial explant.
2. **Difficult or slow to propagate plants**. Micropropagation enables growers to increase the

production of plants that normally propagate very slowly such as narcissus and other bulbous crops.

3. **Introduction of new cultivars** eg. Dutch iris. Get 5 daughter bulbs annually. Takes 10 years for commercial quantities of new cultivars to be built up. Can get 100-1000 bulbs per stem section.

4. **Vegetative propagation of sterile hybrids** used as parent plants for seed production. Eg. cabbage.

5. **Pathology - Eliminate viruses, bacteria, fungi etc**. Use heat treatment and meristem culture. Used routinely for potatoes, carnation, mum, geranium, garlic, gypsophila

6. Storage of germplasm

Generally the only successful method to date is keeping them in refrigerator. Slows down, but does not eliminate, alterations in genotype.

Questions

1. Multiplication of genetically identical copies of a cultivar by asexual reproduction is called
a) Clonal propagation b) Apomixis
c) Vegetative propagation d) None of the above

2. In nature, clonal propagation occurs by …….
a) Vegetative propagation b) Apomixis
c) Both a & b d) None of the above

3. Apomixis is by …….
a) Seed development without meiosis and fertilization **b)** Regeneration of new plants from vegetative parts
c) Both a & b d) None of the above

4. Vegetative propagation is by …….
a) Seed development without meiosis and fertilization **c)Regeneration of new plants from vegetative parts**
b) Both a & b d) None of the above

5. Advantage of tissue culture is/are …….
a) Production of large number of true-totype plantlets from a single plant c) Less space requirement **d) All the above**
b) Less time requirement

6. Stage 0 in tissue culture is
a) Preparative stage b) Initiation of culture
c) Multiplication d) Rooting of shoots

7. Stage 1 in tissue culture is
a) Preparative stage **b) Initiation of culture**
c) Multiplication d) Rooting of shoots

8. Stage 2 in tissue culture is
a) Preparative stage b) Initiation of culture
c) Multiplication d) Rooting of shoots

9. Stage 3 in tissue culture is
a) Preparative stage b) Initiation of culture
c) Multiplication **d) Rooting of shoots**

10. Stage 3 in tissue culture is
a) Preparative stage b) Initiation of culture
c) Multiplication **d) Transplanting**

11. Stage 3 in tissue culture is

www.AgriMoon.Com

a) Preparative stage
b) Initiation of culture
c) Multiplication
d) Transplanting

12. The suitable explant for producing virus free plant is ……..
a) Shoot tip
b) Leaf bit
c) Stem bit
d) None of the above

13. The oxidation of phenolic compounds leached out from the cut surface of the explant in tissue culture leads to ……..
a) Browning of the medium
b) Blackening of the medium
c) Whitening of the medium
d) None of the above

14. The oxidation of phenolic compounds leached out from the cut surface of the explant in tissue culture leads to ……..
a) Browning of the medium
b) Blackening of the medium
c) Whitening of the medium
d) None of the above

15. Browning of medium is a common problem in ……..
a) Adult tissues from woody species
b) Juvenile tissues from woody species
c) Both a and b
d) None of the above

16. The crucial stage in tissue culture is
a) Preparative stage
b) Initiation of culture
c) Multiplication
d) Rooting of shoots

17. In tissue culture, the multiplication is through
a) Callusing
b) Adventitious bud formation
c) Enhanced axillary branching
d) All the above

www.AgriMoon.Com

Lecture 07
Merestem tip culture

1. Introduction

Meristematic tissue (meristos = divisible)
A group of identical cells which are in a continuous state of cell division. Some of the cells from the meristematic tissue stops dividing and exhibit certain changes to become permanent tissues of the plant. This change from meristematic to permanent state is called as differentiation. The rest of the cells in the meristematic tissues persists their meristematic activity. The meristematic tissues are self-perpetuating.

Features of meristematic cells
The meristematic cells may be round, oval, polygonal or rectangular in shape. These cells are arranged closely without intercellular spaces. They have dense cytoplasm with large nucleus. They have smaller vacuoles scattered throughout the cytoplasm. Their cell walls were thin, elastic and made up of cellulose.

2. Classification of meristems

Based on its position (Figure 7.1), meristematic tissues are classified into three types:

- I. Apical meristem
  II. Intercalary meristem
  III. Lateral meristem

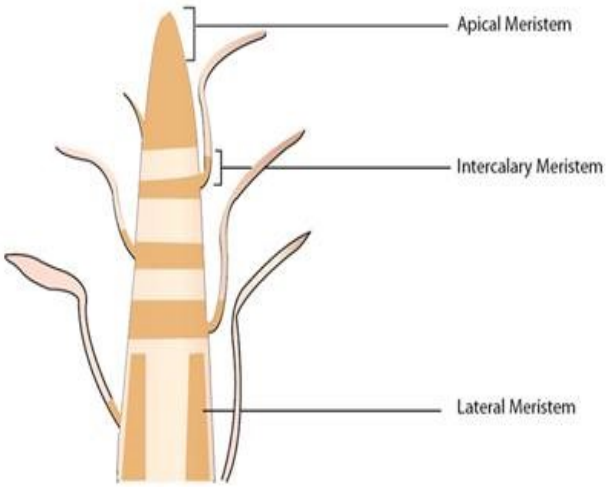


Figure 7.1: Longitudinal section of shoot- showing the positions of meristems

www.AgriMoon.Com

I. Apical meristem

Apical meristem is found at the tips of the roots, stems and branches. It plays a major role in the increase of plant length. It is divided into three zones namely protoderm, procambium and ground meristem. Protoderm gives rise to epidermal tissue. Procambium gives rise to primary vascular tissue and ground stem gives rise to cortex and pith. Apical meristem with two to three leaf primordia constitutes the apex.

II. Intercalary meristem

Intercalary meristems is present in the nodal region and is very prominently found in the monocotyledons, e.g., grasses. The name itself represents that intercalary meristems are found in between the permanent tissues. It is responsible for the elongation of nodes.

III. Lateral meristem

The meristem that is present along the longitudinal axis of stem and root is called lateral meristem, for example, vascular cambium and cork cambium. It produces secondary meristem tissues, which result in the thickening of stem and root.

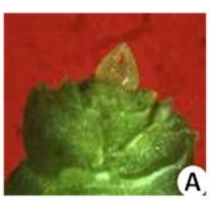
3. Establishment of pathogen free plants

The plants infected with bacteria and fungi can be treated by bactericidal and fungicidal compounds, there is no commercially available treatment to cure virus-infected plants. A large numbers of viruses are not transmitted through seeds. Therefore, it would be possible to obtain virus free plants from infected individuals by using seeds as propagules. However, genetic variation often occurs from the sexually reproduced plants when propagated by seeds. Generally, clonal multiplication of cultivars can be achieved by vegetative propagation. However, where the entire population of the clone is infected the only way to obtain pathogen-free stock is to eradicate the pathogen from vegetative parts of the plants and regenerate full plants from such tissues. Once pathogen free plants are obtained they can be multiplied indefinitely under conditions which would protect them from chance reinfection.

4. *In Vitro* Meristem and shoot-tip culture

4.1. Explant terminology

The scientist who have attempted to recover pathogen-free plants through tissue culture techniques have indiscriminately designated the explants required to initiate cultures as ‘shoot-tip', ‘tip-meristem' and ‘meristem-tip'. The apical meristem of a shoot is the portion lying distal to the youngest leaf primordium, it measures up to about 100µm in diameter and 250µm in length. The apical meristem together with one to three young leaf primordia, measuring 100-500µm, constitutes the shoot-apex (Figure 7.2A and B). Although the chances of eradicating viruses is higher through ‘meristem' culture, in most successful reports virus-free plants have been raised by culturing 100-1000µm long explants which could be according to the above definition is referred as ‘shoot-tip'. To distinguish it from the *in vivo* technique of propagation through shoot-tip cuttings, the term ‘meristem-tip' culture has been preferred for *in vitro* culture of small shoot-tips.



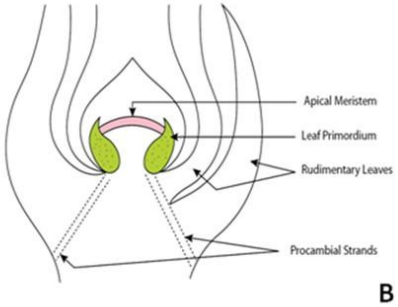


Figure 7.2: A. A shoot-tip explant; **B.** A cross section of shoot-tip meristem

4.2. Reasons for escape of meristem from virus

It is well known that the distribution of viruses in plants is uneven. In infected plants the apical meristems are generally either free or carry a very low concentration of the viruses. In older tissues the virus titer increases with increasing distance from the meristem-tips. The reasons proposed for the escape of meristem from virus invasion are:

1. (a) Viruses readily move in a plant body through the vascular system which is absent in the meristem.
2. (b) The alternative method of cell-to-cell movement of the virus through plasmodesmata is rather too slow to keep pace with the actively growing tip.
3. (c) High metabolic activity in the actively dividing meristem cells does not allow virus replication.
4. (d) The 'virus inactivating systems' in the plant body, if any, has higher activity in the meristem than in any other region. Thus, the meristem is protected from infection.
5. (e) A high endogenous auxin level in shoot apices may inhibit virus multiplication.

5. Factors affecting eradication of virus through meristem tip culture

Culture medium, explant size and incubation conditions affecting plant regeneration from meristem-tip cultures have pronounced effect on virus eradication. Besides, thermotherapy or chemotherapy and physiological stage of the explants also affect virus elimination by shoot-tip culture.

5.1. Culture medium

The nutrients, growth regulators and nature of the medium highly influence the development of virus free plants from meristem tip cultures. Maximum success is achieved from Murashige & Skoog's (MS) medium which promoted healthy, green shoot development compare to other nutrient media. The main reason for the suitability of medium for meristem-tip culture could be the presence of high levels of K^+ and NH_4^+ ions. There is no critical assessment on the role of various vitamins or amino acids but sucrose or glucose is the most commonly used carbon source in the medium, at the range of 2-4%, to raise virus free plants from meristem-tip cultures.

Large meristem-tip explants, measuring 500µm or more in length, may give rise to plants even in the basal medium but generally the presence of an auxin or a cytokinin or both plays a major role in the development of excised apical meristem. In angiosperms, the meristematic dome in the shoot-tip does not synthesize auxin on its own, but it is supplied by the second pair of youngest leaf primordia. Therefore, for development of excised meristem in culture, without the leaf primordia, requires the supply of exogenous auxin. The plants requiring only auxin must have a high endogenous cytokinin level in their meristems. Among auxins, the use of 2,4-D should be avoided which promotes only callusing. NAA and IAA are widely used auxins and NAA being preferred due to better stability. The role of GA_3 is also emphasized by few authors which is suggested to promote better growth and differentiation and suppresses callusing from meristem explants. Both liquid and semi-solid media have been tried for meristem–tip culture but, agar medium is generally preferred.

5.2. Explant size

The survival of the meristem tips, under the controlled condition, is determined by the size of the explant. The larger the explant, the greater are the chances of plant regeneration. However, the survival of the explants can not be treated independent of the efficiency with which virus elimination is achieved that is inversely related to the size of the explant. Thus, explants should be small enough to eradicate viruses and large enough to be able to develop into a complete plant. Besides the size of the explant, the presence of leaf primordia influences the ability of the meristems to form plants. In some plants it is essential to excise shoot meristems with two to three leaf primordia. Smith and Murashige (1970) have suggested that leaf primordia supply auxin and cytokinin to the meristem

necessary for its growth and differentiation. In a culture medium containing essential growth regulators, the excised meristems domes develop bipolar axes very quickly during reorganization (Figure 7.3). Once the root-shoot axis is established further development follows the same pattern as that of seedlings.

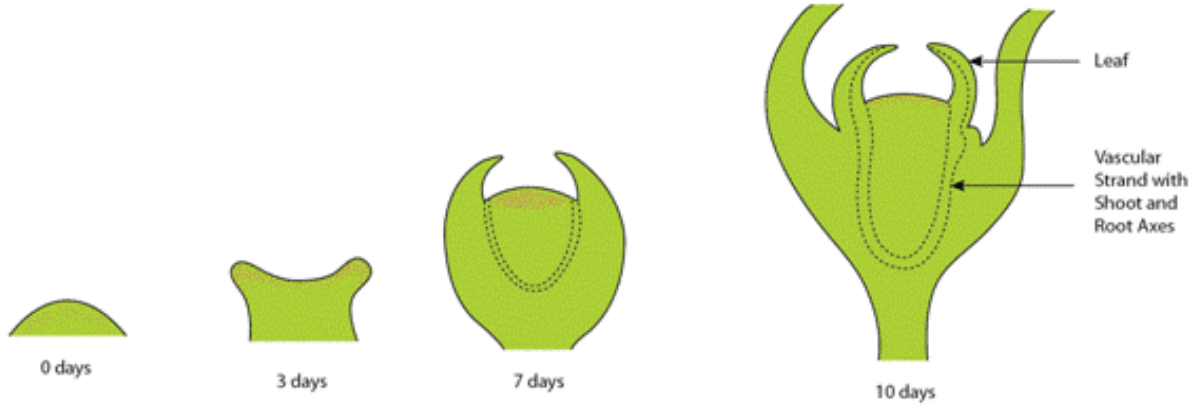


Figure 7.3: Schematic representation of development of bipolar axes by meristem culture

5.3. Storage conditions

Generally, light incubation of meristem tip culture is found better than dark incubation. The light intensity could range from 100 lx to 4000 lx which should increase in succession as the differentiation of meristem explant progresses. There are no clear information on the effect of temperature on regeneration of plants from excised meristem tips. The cultures are normally stored under room temperature (25±2°C) conditions.

5.4. Physiological conditions of the explant

Meristem-tips should be collected from actively growing buds. In few cases, the tips taken from terminal buds proved better than those taken from axillary buds. Seeing the higher number of axillary buds present per shoot, in majority of the reports axillary buds were utilized as explants to increase the overall production of virus-free plants. The time excision of buds is also critical, specially for the trees with periodic growth. For example, in temperate trees the growth of the plant is limited to only a very short period in the spring and afterwards dormancy starts. In such cases, the meristem-tip cultures can be raised during spring only for increased success rate.

5.5. Thermotherapy

Often, apical meristems are not always free of virus and it can't be considered as a universal occurrence. There are certain viruses like, Tobacco Mosaic Virus (TMV), Potato Virus X (PVX) and Cucumber Mosaic Virus (CMV), which invade the meristematic region of the growing tips and interrupts the growth of the meristematic tissue. In such cases also it has been possible to obtain virus-free plants by combining meristem-tip culture with thermotherapy. In this technique, first the mother plants are exposed to heat treatment before excising the meristem-tips or, alternatively, shoot-tip cultures are exposed to high temperature regimes (35°C-40°C) for certain duration (6h to 6 weeks) to obtain virus free plants. In the later case, continuous exposure to very high temperature causes deterioration of the host tissues. The first procedure of treating the mother plant has added advantage where larger explants can be taken from the treated stock and thus, favors relatively higher chances of the tip survival.

5.6. Chemotherapy

Chemotherapy is the treatment of an ailment by chemicals especially by killing micro-organisms. It will not eradicate the virus completely. However, a large number of antibiotics, growth regulators, amino acids, purines and pyrimidines can be tested for inactivation of viruses. A nucleotide analogue ribavirin has been found to be the most efficient viracide for plant viruses. This broad spectrum antiviral agent, effective against both plant and animal, was reported to eliminate PVY, CMV and TMV from tobacco explant cultures, Chlorotic Leaf Spot Virus (CLSV) in apple cultures when incorporated into the medium. Vidarabine (adenine arabinoside) and antiserum are also known to reduce the titre of viruses. The effectivity of the compound may vary with the virus and the host genotype.

5.7. Virus elimination through callus culture

It is a general observation that not all the cells in a calli uniformly carry the pathogen when raised from infected tissues. The two possible reasons for the escape of some cells of a systematically infected callus from virus infection are: (a) virus replication is unable to keep pace with cell proliferation, and (b) some cells acquire resistance to virus infection through mutagenesis. Therefore, it is possible to raise virus-free plants from infected shoot-tip calli. However, genetic instability of cultured cells and lack of plant regeneration in callus cultures of some plants poses the limitations of using calli for virus elimination.

6. Virus indexing

Even after subjecting the meristem-tips to various treatments favoring virus eradication, only a proportion of the cultures yield virus free plants. Therefore, it is required to test all plants, regenerated through meristem-tip or callus cultures, for specific viruses before being used as mother plant to produce virus-free stock. The individual plants consistently showing negative results for virus titre can be marked as ‘virus tested' for specific virus/es and can be released for commercial purposes. The following tests can be performed for virus testing:

i. The simplest test for the presence or absence of viruses in plant tissues is to examine the leaves and stem for the visible symptoms characteristic of the virus.

ii. Another test is the sap transmission test or ‘bioassay test' or ‘infectivity test'. It is a very sensitive test and can be performed at a commercial scale. To perform this, ground the test leaves in equal volume (w/v) of 0.5M phosphate buffer using a mortar and pestle. Leaves of the indicator plant (a plant very susceptible to specific viruses), dusted with 600-grade carborundum, are swabbed with the leaf sap from the test plant. After 5 min the incubated leaves are gently washed with water to remove the residual inoculum. The inoculated indicator plants are maintained in a glasshouse, separate from other plants. It may take several days to several weeks, depending on the nature of virus and the virus titre, for the symptoms to appear on the indicator plants. It is used to detect some viruses and viroids but is a slow process requiring several days to months.

iii. The third method, enzyme-linked immunosorbant assay (ELISA), is more rapid serological test which allows quick detection of important viruses. It relies on the use of antibodies prepared against the viral coat protein, requires only a small amount of antiserum and can be performed with simple equipment. However, it is not applicable to viroids and viruses which have lost their coat proteins

2.1. Micropropagation vs. conventional method of propagation

All living plant cells, irrespective of their nature of specialization and ploidy level, have been shown to regenerate plants *via* organogenesis or embryogenesis. The latter involves a highly specialized mode of development that normally occurs only inside the seed, under the cover of several layers of parental tissues. Consequently, the observation of developing embryos and their isolation in intact and living conditions for experimental studies have been extremely difficult. *In vitro*production of embryos from somatic and gametic cells has opened up the possibility of obtaining large numbers of embryos of different stages, enabling investigations on cellular, genetic and physiological control of

embryogenesis (induction, pattern formation, organ differentiation and maturation). *In vitro* expression of cellular totipotency and other techniques of plant tissue culture have also facilitated and/ or accelerated the traditional methods of plant improvement, propagation and conservation.

2.2. Micropropagation vs. vegetative propagation

The vegetative propagation has been conventionally used to raise genetically uniform large scale plants for thousands of years. However, this technique is applicable to only limited number of species. In contrast to this, micropropagation has several advantages which are summarized here:

i. The rapid multiplication of species difficult to multiply by conventional vegetative means. The technique permits the production of elite clones of selected plants.
ii. The technique is independent of seasonal and geographical constraints.
iii. It enable large numbers of plants to be brought to the market place in lesser time which results in faster return on the investment that went into the breeding work.
iv. To generate disease-free (particularly virus-free) parental plant stock.
v. To raise pure breeding lines by *in vitro* haploid and triploid plant development in lesser time.
vi. It can be utilized to raise new varieties and preservation of germplasm
vii. It offers constant production of secondary medicinal metabolites.

Trouble shooting

- Few explants exude dark colored compounds, like phenols, pigments etc which leach into the medium from the cut ends of the explant. It results in the browning of tissues and the medium as well. The browning of medium is associated with poor culture establishment and low regeneration capacity of the explants. This can be overcome by:

i. minimizing the wounding of explants during isolation and surface disinfection to reduce this browning response.
ii. washing or incubation of explants for 3-5 hrs in sterile distilled water to remove phenolics responsible for browning of medium or explants.
iii. frequent subculture of explants with excision to fresh medium at regular intervals.
iv. initial establishment of cultures in liquid medium and later transfer to the semi-solid medium.
v. culture of explants on porous substrate or paper bridges.
vi. addition of activated charcoal (AC) or polyvinylpyrrolidone (PVP) for adsorbtion of phenolics.
vii. antioxidants like ascorbic acid, citric acid etc. can also be used to prevent browning of tissues in culture.

• Appearance of vitrified tissues (hyperhydricity), a physiological disorder occurring in the *in vitro* cultures due to which the tissues look transparent and fluffy resulting from excessive intake of water. Hyperhydricity can be caused by a high concentration of cytokinin or low concentration of gelling agent or high water retention capacity of explants if the container is tightly closed.

• Loss of regeneration ability in long-term cultures due to epigenetic variations (temporary variations) and culture aging, including transition from juvenile to mature stage. Epigenetic variation are phenotypic temporary variations which disappear as soon as the culture conditions are removed.

• Genotypic variations are also seen in the cultures, therefore, cytological, biochemical and molecular analyses are required to confirm clonal fidelity of *in vitro* regenerants. Besides, morphological and physiological testing is also required to remove undesired genetic variability.

1. Introduction

Axillary buds are usually present in the axil of each leaf and every bud has the potential to develop into a shoot. In nature these buds remain dormant for various periods. The species with strong apical dominance show the growth of axillary buds into shoot only if the terminal bud is removed or injured. The phenomenon of apical dominance is regulated by the interplay of growth regulators. The application of cytokinin to the axillary buds can overcome the apical dominance effect and

stimulate the lateral buds to grow rapidly in the presence of terminal buds. If the exogenous growth regulator diminishes the lateral shoot stop growing.

2. Rate of shoot multiplication

In plant tissue culture, the rate of shoot multiplication can be determined by enhanced axillary branching. Due to continuous availability of cytokinin, the shoots formed by the bud, a priori present on the explant (nodal segment or shoot-tip cutting), develops axillary buds which may grow directly into shoots. This process may be repeated several times and the initial explant transformed into a mass of branches. There is a limit to which shoot multiplication can be achieved in a single passage, after which further axillary branching stops. At this stage, if shoots are excised and planted on a fresh medium of same composition, the shoot multiplication cycle can be repeated. This process can go on indefinitely, and can be maintained throughout the year independent of the season and the region.

In some plants, it may not be possible to break apical dominance by the application of growth regulator compositions, and the bud present a priori on the initial explant grows into an unbranched shoot. The rate of shoot multiplication in such cases would depend on the number of nodal cuttings that can be excised from the newly developed shoot at the end of each passage. With this alternative method of enhanced axillary branching, 6-7 fold shoot multiplication every 4-6 weeks could be achieved in the plants with strong apical dominance.

3. Factors affecting axillary shoot proliferation

i. Effects of season on culture establishment

The extent of contamination as well as bud-break is highly dependent on the season. The cultures initiated during spring season (January to April) shows best response not only in terms of the frequency of bud-break but also in the vigor of the shoots with least contamination rate. Since, summer (May-August) is the period that concurs with rainy season in certain regions like India, the cultures are prone to infection. By winter the shoots become old and it is difficult to break the dormant state of the buds.

ii. Effect of carbon source on shoot proliferation

In cultured plant tissues, a continuous supply of carbohydrate from the medium is essential which are needed for growth and organized development of the plant and are necessary as a source of energy and carbon skeletons for biosynthetic process. For shoot induction from axillary buds, three carbon sources, sucrose, glucose and maltose are utilized in maximum plant tissue cultures at a fixed concentration of 30 g l^{-1} . Of these, sucrose is the most commonly used carbohydrate for plant tissue cultures and most culture media have it as the sole carbohydrate source. It favors higher growth of shoot, number of nodes per shoot and the rate of shoot multiplication compare to maltose and glucose. Sucrose is easily recognized and hydrolyzed by cell wall bound invertase into more efficiently utilizable forms of sugars, glucose and fructose which are incorporated into the cells. Glucose, derived from sucrose hydrolysis, is more accessible to the cultured tissues than glucose derived by maltose hydrolysis, due to a rapid sucrose hydrolysis but a slow maltose hydrolysis in the media.

iii. Effect of growth regulators on shoot proliferation

In general, cytokinins favors shoot proliferation and auxins favors root formation. In *S. acmella,*nodal explants bearing two opposite axillary buds were when cultured on MS basal medium or basal medium supplemented with BAP, Kinetin or 2-iP at 3 µM concentration, the frequency of bud-break was appreciable in basal medium but incorporation of BAP to the basal medium has further improved the incidence of bud-break and promoted multiple shoot formation (2 shoots/explant). While the least bud-break was observed on Kinetin supplemented medium and 2-iP was noticed to be inhibitory for axillary bud proliferation. The addition of a low concentration of GA_3 to the BAP supplemented medium further promoted multiple shoot formation. On the other hand, single shoot with long internodes was developed from axillary buds in cultures when NAA

was added to BAP containing medium. The frequency of bud-break varied with the concentration of the BAP and at its optimum level of 5 μM, 10-fold shoot multiplication occurred every 5 weeks.

4. Axillary shoot proliferation vs adventitious shoot proliferation

i. The axillary shoot proliferation is the most popular approach to clonal propagation of crop plants because the cells of the shoot apex are uniformly diploid and are least susceptible to genotypic changes under culture conditions.

ii. Chimeras, whose breakdown is common during adventitious bud proliferation, are perpetuated in shoot-bud culture and, thus, the cause for change in ploidy sometimes. While axillary shoot proliferation favours genetically uniform plant formation.

iii. Moreover adventitious bud formation and callusing methods require denovo differentiation of shoot-buds which is not always possible.

iv. Further, the axillary shoot proliferation is comparatively a quicker method of shoot multiplication as pre-existing meristem only proliferate into shoots, thus, reducing the time required to form de novo meristem formation.

5. Axillary shoot proliferation vs conventional method of propagation

i. The conventional method of vegetative propagation by stem cuttings utilizes the ability of axillary buds to take over the function of main shoot in the absence of a terminal bud. However, the number of cuttings that can be taken from a selected plant in a year is extremely limited because in nature the vegetative growth is periodic. In *in vitro* conditions, axillary shoot proliferate irrespective of seasons and regions.

ii. A minimal size of cuttings required in conventional methods is around 24-30 cm in order to establish a plant from it. Thus, it may restrict the multiplication of plants if the stock of parent plant is limited or if the species is endangered.

iii. With axillary shoot proliferation, minimum cutting size required is <1cm, thus, it favours large scale multiplication even with the limited sample.

iv. With the axillary shoot proliferation method, juvenile nodal cuttings are made available throughout the year that helps to maintain faster rate of multiplication compare to conventional methods of vegetative propagation where juvenile phase is short lived and with mature cuttings it is difficult to establish propagation as the buds in the axil under go dormancy.

Lecture 08
Micropropagation – Problems - Applications - Advantages and limitations

Problems

1) Microbial contamination

Bacterial and fungal contamination in culture do not allow propagules to grow and contaminated cultures have to be usually discarded. Such a problem can be overcome by growing the donar plant in growth chamber, by effective sterilization of explants, by performing inoculation in the laminar air flow cabinets and by using sterlised surgical
instrument. Fumigation of inoculation with dilute formaldehyde solution helps to minimise this problem.

2) Callusing

Callus formation is highly undersirable as it often effects the normal development of shoots and roots and may lead to variability among the regenerated plants

Additon of tri-iodo-benzoic acid, flurogauicinol and flurorizin into the culture medium (or) reduction of inorganic salt concentration helps in overcoming this problem

3) Tissue culture induced variation

The Micro propagation plants exhibit genetic (or) epigenetic variations which may be a major problem in getting true to type plants. It can be controlled by careful selection of initial explant, tha t is selecting meristems and controlling the cultural environment favouring slow multiplication rates

4) Browning of medium

In may species phenolic substances leach into the medium from the cut surfaces of explant. These phenolics turn brown on oxidation and lead to browning (or) blackening of medium and or explants.

These oxidation products are detrimental to the cultures as they cause necrosis and eventually death of the cultures This problem is very common incase of woody species particularly when explants are taken from mature trees. This problem can be overcome by one of the following ways.

i. Frequent subculture (every 3-7 days) of explants on Agar medium

ii. A brief period (usually 3-10 days) of culture in liquid medium is effective in many species Eg:- Apple, Eucalyptus

iii. Antioxidants like citric acid (150 mg / lit) may be used to check the oxidation of poly - phenols
iv. Adsorbents like activated charcoal (0.5-2g/lit) (or) PVP (poly vinyl pyrrolidon) may be used to adsorb the poly phenols secreted in to the medium

v. Culture incubation in dark may be helpful since light enhances polyphenol oxidation as well as polyphenol bio -synthesis.

5) Vitrification

Some shoots developed *in vitro* appear brittle glassy and water soaked. This is called vitrification (or) hyper hydration. The plants appear abnormal because of abnormal leaf morphology. Poor photosynthetic efficiency malfunctioning of stomata, reduced epicuticular waxes. It can be reduced by reducing the relative humidity in culture vessels. Reducing the
cytokinins level (or) NH4 levels (or) salt concentration in the medium, addition of flurorizin,

flurorogluc inol (or) $Cacl_2$ in medium etc

6) Vulnerability of micro propagation plants to transplantation shocks

High mortality rates upon transferring the tissue culture derived plants to soil continuously to be a major bottle neck in micro propagation of many plants species.

Conservation of moisture by creating high humidity around the plant, partial defoliation, application of antitransperants have met with good suc cess.

Advantages:-

1. To get genetically uniform plants in large number
2. Only a small explant is enough to get millions of plants with extremely high multiplication rate
3. Rapid multiplication of rare and elite genotypes
4. This technique is possible alternative in plants species which do not respond to conventional bulk propagation technique
5. In plants with long seed dormancy micro propagation is faster than seed propagation
6. Useful to obtain virus free stocks
7. In dioecious species plants of one sex is more desirable than those of other sex
8. Eg:- Male asparagus and Female papaya, In such cases plants of desired sex can be selectively multiplied by this technique
9. This technique is carried out through out the year independent of seasons
10. undesirable juvinile phase associated with seed raised plants does not appear in micro propagation plants of some species
11. Considerable reduction in period between selection and release of new variety.
12. Maintenance of parental lines (male sterile lines especially) for the production of F_1 hybrid seed.
13. Facilitates speedy international exchange of plant materials
14. Meristems have been identified as an excellent material for germplasm preservation of some species
15. In case of ornamentals tissue culture derived plants give better growth, more flowers and less fall out

Limitations

1. This technique has limited application because of high production cost
2. At each stage the technique has to be standardized
3. Suitable techniques of micro propagation are not available for many crop species

4. Somaclonal variation may arise during *in vitro* culture especially when a callus phase is involved eg- banana
5. Vitrification may be problem in some species
6. Browning of medium is a problem in woody **(Adult trees)** perennials
7. Requires highly advanced skills
8. requires a transitional period before the plants are capable of independent growth
9. The plants obtained are photosynthetically not self sufficient
10. The plantlets are susceptible to water losses in external environment and they have to be hardened to the external atmosphere
11. Aclamatisation is difficult process to get high percentage of suruvial of plants
12. Continuous propagation from same material for many generations may lead to many off types in culture
13. This is available for lab scale not for commercial scale
14. Inspite of great care taken during culture there are chances of contamination by various pathogens which could cause vary high losses in a short time

Applications:-

1) Micro propagation of a hybrid has the greatest multiplication advantage since it can be result in large number of elite plants from a very small tissue clump taken from the hybrid plant.
2) Maintainance of inbred lines for producing F1 hybrids
3) Maintainance of male sterile genotypes of wheat and onion are useful in hybridization.
4) selective propagation of dioecious plants Eg:- female plants of papaya, male plants of Asparagus
5) Multiplication of particular heterozygous superior genotype with increased productivity Eg:- oil palm
6) shoot cultures of some species are maintained as slow growth culture for germ plasm conservation
7) Rapid production of disease free material
8) Tissue culture can be used to minimise the growing space in commercial nurseries for maintenance of shoot plant

Micro-propagation- Definition, methods, stages of micro-propagation and its significance; Advancement and future prospects of micro-propagation. Micropropagation - Axillary bud proliferation approach- Shoot tip and meristem culture;
Definition: Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation.

The in vivo clonal propagation is often difficult, expensive and even unsuccessful. Tissue culture method offers an alternative way of clonal propagation which is popularly known as micro-propagation.

Here in this method a multiple number of miniatures of vegetative shoots are produced from a clone within a short time and space (Fig. 18.8). Use of tissue culture for micro-propagation was initiated by G. Morel (1960) who found this as the only commercially viable approach for orchid propagation.

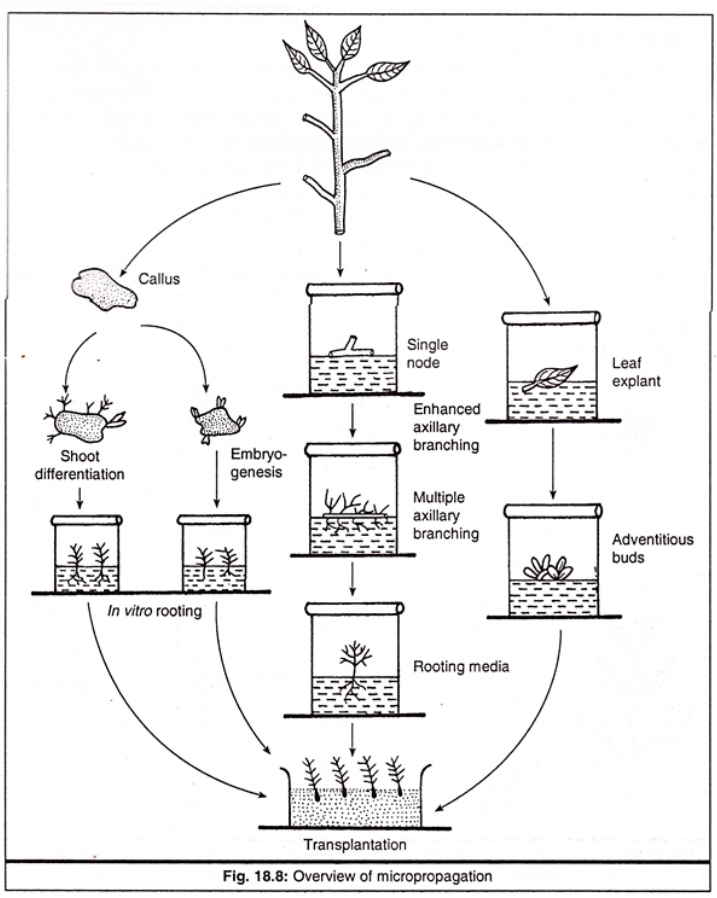


Fig. 18.8: Overview of micropropagation

The different techniques of single cell and protoplast culture also enable thousand plants to be derived within a short space and time. The products of these rapid vegetative propagation can also be regarded as clones when it is established that the cells they comprise are genetically identical.

Lecture 09

Methods of Micro-Propagation:

(i) Multiplication by Meristematic Tissue of Axillary and Apical Shoots:

Axillary and apical shoots contain quiescent or active meristems depending on the physiological state of the plant. When these shoot tips are cultured on a basal medium containing no growth regulators, these typically develop into single seedling like shoots with strong apical dominance (Fig. 18.9A-B).

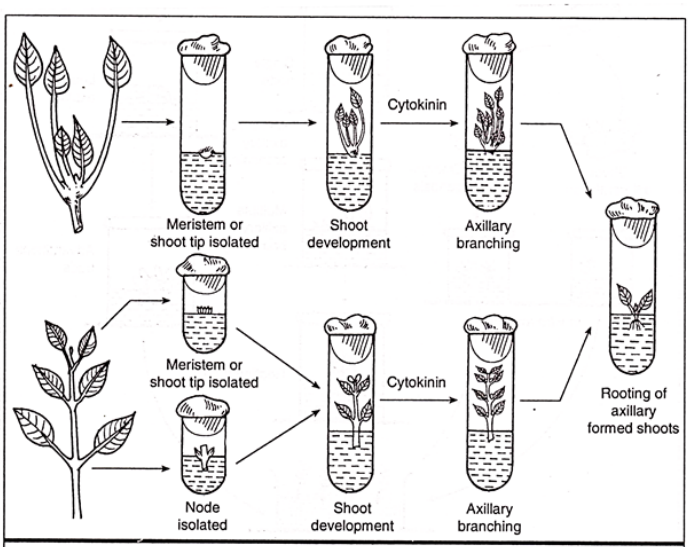


Fig. 18.9A: Scheme showing the *in vitro* micropropagation of plants by the axillary bud method. Upper row: as applied to rosette plants; bottom row: applied to plants which elongate

On the contrary, when the shoots of the same explant material are grown on culture media containing cytokinin, axillary shoots develop precociously which proliferate to form clusters of secondary and tertiary shoots. These clusters when subdivided and transferred onto fresh medium again these will form similar clusters.

This subdivision process may be continued indefinitely when provided with basic nutrients. About 5-10 multiplication rates on 4-8 weeks of micro-propagation cycle may ultimately lead to extremely impressive clonal propagation range of 0.1-3.0 x 10^6 within a year.

(ii) Multiplication by Adventitious Shoots:

Axillary buds are "preformed meristems" present at the leaf axils, whereas adventitious buds may arise from any plant structures and this regeneration is often dependent on the presence of organised plant tissue. The explants may be stems, internodes, leaf blades, cotyledons, root elongation zone, bulb, corms, tubers, rhizomes, etc.

If these explants are induced by using appropriate level of growth regulators in the medium they will form the meristematic zone which regenerates multiple shoots on a suitable culture medium. High number of adventitious shoots may arise only from a single epidermal cell.

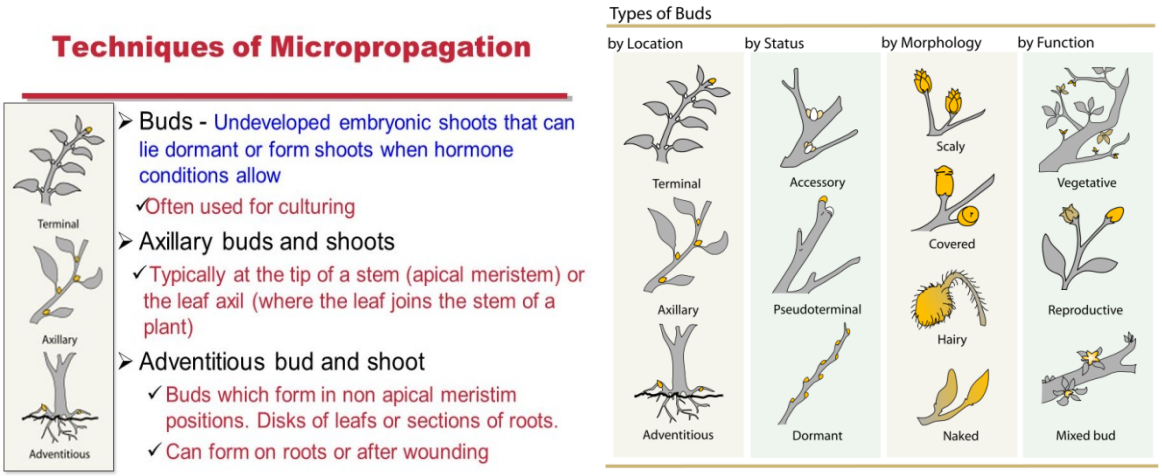
Continuous propagation by adventitious shoot proliferation from bulbs and corms can be achieved by cultivating two vertically split piece of shoot bases. Clusters of shoots may develop from the abaxial surfaces of developing leave and scales. Trimming of the shoot apices is a good approach which has been found to ensure continuously productive cultures of different hybrid plants for indefinite period.

(iii) Multiplication by Adventitious Embryo Formation:

It is another useful approach followed for many important plant species. Adventitious embryos can arise directly from a group of cells within the original explants or from primary embryoids.

There are many plant species which develop embryos in vivo from diverse kind of explants e.g. the orchid leaf tips produce large number of embryoids whereas Citrus and Mangifera have shown to

develop polyembryos from nucellar tissue.
These adventitious embryoids are diploid in nature and can be used as clonal material for micro-propagation. In in vitro similarly the adventitious embryos originated from different explants can be good materials for clonal propagation.
(iv) Multiplication through Callus Culture:
Direct plantlet formation from the explants in culture is more desirable in case of micro- propagation or clonal propagation. But shoot formation may occur through organogenesis or embryogenesis from the callus produced from the explant.
The limitation of this system is that the callus cells are not genetically stable so it cannot be called as a single clone and this process is more time consuming. Also the plant regeneration capacity may decline due to the genetic unstable condition.
In case of some plant species genetically stable calli have also been derived, in these types of calli slow growing meristematic zones are formed from the peripheral layer. The meristematic layers invariably comprise of diploid cells expressing totipotency. These types of calli can be subdivided into smaller parts and which may produce multiple shoots from each.



Stages of Micro-Propagation:
The process of micro-propagation or clonal propagation is a complicated process which can be subdivided into four prominent stages of operation:
Stage 0:
This is the initial step of micro-propagation in which stock plants has to be j grown under controlled condition before using for culture initiation.
Stage I:
The preparation of explants from stock plants is followed by its establishment j in a suitable culture medium.
The steps involved in this stage are:
(a) Explant isolation,
(b) Surface sterilisation,
(c) Washing,
(d) Establishment of explant on appropriate culture medium.
Stage II:
This stage involves the multiplication of shoots or rapid somatic embryo formation using a defined culture medium.
Various Approaches followed for Micro-Propagation Include:
(i) Multiplication through the growth and proliferations of meristems excised from apical and axillary shoot of the parent plant.
(ii) Induction and multiplication of adventitious meristems through processes of organogenesis or somatic embryogenesis directly on explants.

(iii) Multiplication of calli derived from any kind of explant and subsequently shoots development either through organogenesis or embryogenesis. The harvest cycle generally takes 4-8 weeks. Either the shoots are marketed directly or carried over to the next stage for further development.

Stage III:

Shoots obtained from stage II are transferred to the next rooting or storage medium. These shoots are directly established in soil as micro-cuttings to develop roots. The shoot handling at this stage differs from species to species.

When the shoots are directly transferred to soil then the following procedures are to be maintained:

(i) Each shoot should be rooted individually,

(ii) Hardening of the shoots to increase their resistance,

(iii) Allowing the plants capable of autotrophic development rather than the heterotrophic development in culture,

(iv) Fulfilling the requirements for breaking dormancy.

Stage IV:

Transfer of plantlets to sterilized soil for hardening under greenhouse environment is achieved. This step is to ensure the successful transfer of the plantlets of stage III or un-rooted shoot apices of stage II into the suitable compost mixture or soil in pots under controlled condition of light, temperature, humidity.

For marketing sometimes these plat lets are established in an artificial growing medium such as soil-less mixes, Rockwood plugs or the sponges. It takes 4-16 weeks for marketing of the finished products.

Advantages of Micro-Propagation:

1. The technique of micro-propagation is an alternative approach to conventional methods of vegetative propagation, which has the enhanced rate of multiplication.
2. A million of shoot tips can be obtained from a small, microscopic piece of plant tissue within a short period of time and space.
3. The advantage in this type of propagation is that as shoot multiplication usually has a short cycle (2-6 weeks) and each cycle results in logarithmic increase in the number of shoots.
4. This method of propagation is more advantageous in case of bulb or corm producing plants, because mini-tubers or mini-corms for plant multiplication are available throughout the year irrespective of season.
5. Smaller size of propagules are advantageous for storing and transporting as it takes lesser space.
6. The propagules can be maintained in soil-free environment which facilitates their storage on a large scale.
7. Stocks of germplasm can be maintained for many years using this method of propagation.
8. This method is more applicable where disease free propagules are wanted. This in vitro technique helps to raise pathogen free plant and to maintain them.
9. Micro-propagation is very useful in case of dioecious plants, because there the seed progeny yield is 50% male and 50% female, but this technique helps to get the progeny according to the desired sex.
10. A major advantage of micro-propagation happens to be the minimum growing space required in commercial nurseries. Thousands to millions of plantlets can be maintained within the culture vials. This is specially useful for maintaining the horticultural species.
11. This method is more helpful in case of slow growing plants where the seeds are produced after a long term and the seeds are the only propagule. This method can overcome the difficulty to obtain the propagules.
12. Through seed production genetically uniform progeny is not possible always. But the micro-propagation method will help to maintain the genetic uniformity in the propagules.

13. Micro-propagation is one of the finest ways of plant multiplication by in vitro technique of plant tissue culture. The newer tissue material obtained through r DNA technology or haploid culture or somatic hybridization can be the source of tissue material for micro-propagation, as it is the easiest method for obtaining the multiple propagules.

Commercial Uses of Micro-Propagation:

Micro-propagation of orchids demonstrated profit all over the world, besides orchid about 600 species of other ornamental plants have been successfully cloned and some of them are commercially exploited (Chrysanthemum, Carnation, Gerbera and Anthurium), list of names is increasing day by day, the advantage is rapid cloning of selected colour individuals.

Except the horticultural flowering plants, a large number of forest trees, fruit trees, oil producing plants (Eucalyptus, Oil-palm), vegetables and many edible crops like peach, apple, pear, cherry, apricot and palm have been cloned and put into use commercially (Fig. 18.10).

Breeding and selection by sexual hybridization is a very slow procedure because of long generation time and they are difficult to propagate vegetatively. So the in vitro cloning of more than 100 woody species over a wide range of families has been successfully achieved.

Forest trees are important sources of our biodiversity and also they provide food, fuel, construction and industrial products for us.

These resources are diminishing at an unprecedented rate. Large scale, cost-effective and nursery-friendly in vitro technique like micro-propagation protocols have been developed for selective forest trees; about 70 forest tree species of Angiosperm and about 30 forest tree species of Gymnosperm.

The most important aspect of commercial micro-propagation is the economics involved and the unit cost of a plantlet. Research achievements in the field of micro-propagation are not always economically viable and not accepted by commercial houses. The investment in commercial tissue culture business will depend to a large extent on cost of the laboratory set up, type of plant to be propagated and the skill involved.

The commercial nursery man should start with those crop species for which published methods are available and also it is essential that the grower must have some training in tissue culture and plant husbandry. Another approach followed in micro- propagation is to automate it at its various stages.

In this connection the bioreactors are being used for large scale multiplication of somatic embryos, shoots and bulbs. Automatisation have also been used for subculture of shoots during stages III and IV of micro-propagation. This reduces the cost of labour component in micro-propagation.

Trouble shooting

- • Few explants exude dark colored compounds, like phenols, pigments etc which leach into the medium from the cut ends of the explant. It results in the browning of tissues and the medium as well. The browning of medium is associated with poor culture establishment and low regeneration capacity of the explants. This can be overcome by:
-
- i. minimizing the wounding of explants during isolation and surface disinfection to reduce this browning response.

  ii. washing or incubation of explants for 3-5 hrs in sterile distilled water to remove phenolics responsible for browning of medium or explants.

  iii. frequent subculture of explants with excision to fresh medium at regular intervals.

  iv. initial establishment of cultures in liquid medium and later transfer to the semi-solid medium.

  vi. culture of explants on porous substrate or paper bridges.

  vi. addition of activated charcoal (AC) or polyvinylpyrrolidone (PVP) for adsorbtion of phenolics.

  vii. antioxidants like ascorbic acid, citric acid etc. can also be used to prevent browning of tissues in culture.

• Appearance of vitrified tissues (hyperhydricity), a physiological disorder occurring in the *in vitro* cultures due to which the tissues look transparent and fluffy resulting from excessive intake of water. Hyperhydricity can be caused by a high concentration of cytokinin or low concentration of gelling agent or high water retention capacity of explants if the container is tightly closed.
• Loss of regeneration ability in long-term cultures due to epigenetic variations (temporary variations) and culture aging, including transition from juvenile to mature stage. Epigenetic variation are phenotypic temporary variations which disappear as soon as the culture conditions are removed.
• Genotypic variations are also seen in the cultures, therefore, cytological, biochemical and molecular analyses are required to confirm clonal fidelity of *in vitro* regenerants. Besides, morphological and physiological testing is also required to remove undesired genetic variability.

Lecture 10

Somatic embryogenesis

The sporophytic generation of a plant is initiated with the zygote, which is the initial cell (product of gamete fusion) that bears all the genetic information to construct the adult individual. In angiosperms, the zygote divides transversally, resulting in two cells. One of them, the apical cell, is small and dense with an intense activity of DNA synthesis. Further ordinal divisions of this cell give rise to the embryo head that will be the new plant. The other resultant cell (basal cell) is a large and highly vacuolated one that will confirm the suspensor complex, which plays an important role during the early stages of the young embryo. *In vivo*-produced somatic embryos generally follow the same pattern; however, they are not initiated from a zygote, but from a somatic cell as occurs in the polyembryony.

Somatic embryos

Somatic embryos are bipolar structures with both apical and basal meristematic regions, which are capable of forming shoot and root, respectively. A plant derived from a somatic embryo is sometimes referred to as an "embling".

Somatic embryos vs zygotic embryos and their advantages

Somatic embryos are structurally similar to zygotic embryos found in seeds and possess many of their useful features, including the ability to grow into complete plants. However, somatic embryos differ in that they develop from somatic cells, instead of zygotes (i.e., fusion product of male and female gametes) and thus, potentially can be used to produce duplicates of a single genotype. Since the natural seed develops as a result of a sexual process in cross-pollinating species, it is not genetically identical to one single parent. In contrast, somatic embryo develops from somatic cells (non-sexual) and does not involve sexual recombination. This characteristic of somatic embryos allows not only clonal propagation but also specific and directed changes to be introduced into desirable elite individuals by inserting isolated gene sequences into somatic cells. This bypasses genetic recombination and selection inherent in conventional breeding technology. If the production efficiency and convenience comparable to that of a true seed are achieved, somatic embryos can be potentially used as a clonal propagation system.

Somatic embryogenesis can be initiated by two mechanisms: directly on explanted tissues, where plants are genetically identical (clonation), and indirectly from unorganized tissues (callus). Propagation by indirect embryogenesis carries the risk of producing
plants that may differ genetically from each other and from the parental plant. It is thought that the occurrence of genetic variability within tissue cultures may partly originate from cellular changes that are induced during culture. The genetic variability associated with tissue and cell culture is named "somaclonal variation" and represents an opportunity where selection pressure can be imposed to isolate unique genetic forms of a clone. The ability to recover plants from single cells has made possible the genetic improvement *in vitro*. Plant tolerance to abnormal temperatures, herbicides, fungal toxins, high levels of salt, etc., can be obtained by exposing cell cultures to a selective agent. The most important advantages of the cell- and tissue-culture tools used in plant breeding are:

- Freedom from the effects of the natural environment.
- The ability to handle large numbers of individuals (cells) in very small spaces.
- Creation of variation within cultures. Variability usually occurs spontaneously; however, it can be induced by mutagenic agents.
- Genetic variability can be created deliberately in cultured cells by using genetic-

engineering techniques.

Practical applications of somatic embryogenesis Clonal propagation

Somatic embryogenesis has a potential application in plant improvement. Since both the growth of embryogenic cells and subsequent development of somatic embryos can be carried out in a liquid medium, it is possible to combine somatic embryo-genesis with engineering technology to create large-scale mechanised or automated culture systems. Such systems are capable of producing propagules (somatic embryos) repetitively with low labour inputs. In this process of repetitive somatic embryogenesis (also referred to as accessory, adventive, or secondary somatic embryogenesis) a cycle is initiated whereby somatic embryos proliferate from the previously existing somatic embryo in order to produce clones.

Cloning zygotic embryos for repetitive somatic embryogenesis

A wide range of soybean genotypes have been tested for their ability to undergo auxin-stimulated somatic embryogenesis during cloning of zygotic embryos (Barwale *et al*. 1986; Komatsuda and Ohyama 1988). All of them are reported to form somatic embryos provided appropriate nutrients are provided in the medium. The role of genotypes in conferring regeneration capacity is further supported by studies on zygotic embryo cloning of wheat, rice and maize. Analysis of various cultivars demonstrated that the regeneration capacity of these crops was directly affected by non-additive, additive and cytoplasmic factors. However, the genotype which has the capacity to undergo repetitive somatic embryogenesis can be back-crossed to elite lines in order to transform the latter with capacity for high regeneration of somatic embryos. Such a type of transformation could play an important role in plant breeding since through in vitro techniques high quality somatic embryos have been produced in 80 species of tropical crops.

Raising somaclonal variants in tree species

Embryos formed directly from pre embryogenic cells appear to produce relatively uniform clonal material, whereas the indirect pathway generates a high frequency of somaclonal variants. Mutation during adventive embryogenesis may give rise to a mutant embryo which on germination would form a new strain of plant. Nucellar embryos, like shoot tips, are free of virus and can be used for raising virus-free clones, especially from some tree species (e.g., poly embryonate Citrus) where shoot tip culture has not been successful. For clonal propagation of tree species, somatic embryogenesis from nucellar cells may offer the only rapid means of obtaining juvenile plants equivalent to seedlings with parental genotype. Clonal propagation through somatic embryogenesis has been reported in 60 species of woody trees representing 25 families. Somatic embryos with genotype of a selected elite parent are potentially convenient organs for cryopreservation and germplasm storage.

Synthesis of artificial seeds

There development of methods for encapsulation of somatic embryos to enable them to be sown under field conditions as „synthetic" or „artificial" seed is gaining importance. Research programmes on production of artificial seeds via somatic embryogenesis in respect of commercially important crops would not only contribute to increased agricultural production, but also add to our basic knowledge of the regulatory mechanisms which control plant growth and differentiation.

Source of regenerable protoplast system

Embryogenic callus, suspension cultures and somatic embryos have been employed as sources of protoplast isolation for a range of species. Cells or tissues in these systems have demonstrated the potentiality to regenerate in cultures and, therefore, yield protoplasts that are capable of forming whole plants. Embryogenic cultures are especially

valuable in providing a source of regenerable protoplasts in the graminaceous, coniferous and citrus species. Attempts to achieve regeneration of callus or even sustained divisions in mesophyll-derived protoplasts of Gramineae proved unsuccessful until Vasil and Vasil (1980) turned to embryogenic cultures obtained from immature embryos of pearl millet (*Pennisetum purpureum*) as the source of protoplasts. Protoplasts from these cultures were induced to divide to form a cell mass from which embryoids, and even plantlets, regenerated on a suitable nutrient medium. Similar success was subsequently reported by other workers with embryogenic suspension of *Panicum maximum*, *Pennisetum purpureum*, *Oryza sativa*, *Saccharum officinarum*, *Lolium perenne*, *Festuca arundinaceae* and *Dactylis glomerata*. Among cereals, in Gramineae, the development of a protoplast regeneration system for maize has been especially challenging. Rhodes *et al*. (1988) were the first to raise protoplast derived maize plantlets from embryogenic cultures which, however, proved sterile. Later, Shillito *et al*. (1989) initiated embryogenic callus cultures from immature zygotic embryos of an elite inbred maize line B73, which yielded protoplasts that regenerated to form fertile plants. The protoplast-derived plant on crossing with pollen from a seed-derived plant produced viable seeds which germinated normally. Using similar techniques, Prioli and Sondahl (1989) obtained fertile maize plants from embryogenic cell suspension protoplasts of a line (Cat 100-1) adapted to tropical regions.

Embryogenic citrus suspension cultures also provide protoplasts that can be used in the production of interspecific and intergeneric somatic hybrid plants. Equal success can be obtained with protoplasts isolated from nucellus-callus culture of this plant. Ability to isolate protoplasts from embryogenic cultures has had a large impact on their *in vitro* culture of forest trees, e.g., *Pinus taeda*, *Picea glauca*, *P. mariana*, *Pseudotsuga menziesii*, *Abies alba*, *Santalum album* and *Liriodendron tulipfera*. Somatic embryos induced on the protoplast-derived calli also germinate to form plantlets which finally establish in the soil.

Genetic transformation

In seed embryogenesis, zygotic embryos are seated deep inside the nucellar tissue. They live in a protected environment besides being genetically heterogeneous. On the contrary, somatic embryos remain virtually unprotected and more or less give rise to genetically uniform plants. The advent of leaf-disc transformation systems has made it possible to successfully engineer species (*Nicotiana tabacum*, *Medicago sativa*) in which, tissues are capable of regeneration *via* somatic embryogenesis.

In these species, isolated single cells can be transformed in cultures and grown on a selection medium (nutrient medium containing an antibiotic, kanamycin) to callus colonies which eventually form somatic embryos on removal of auxin from this medium (Chabaud *et al*., 1988). Since the callus phase seems essential in this type of indirect somatic embryogenesis, the possibility of chimeric embryos arising from transformed and non-transformed tissues cannot be ruled out. Therefore, the callus phase can be bypassed through a process of repetitive somatic embryogenesis. McGranahan *et al*. (1990) used repetitive embryogenesis for *Agrobacterium*-transformed walnut (*Juglans regia* L.) cells and obtained multiple crops of somatic embryos without employing the callus phase.

There is also evidence to show that repetitive embryos originate from single epidermal or subepidermal cells which can be readily exposed to *Agrobacterium*. Thus, the transformation technique applied to a primary somatic embryo, instead of a zygotic embryo, should give rise to totally transgenic somatic embryos. Repetitive embryogenesis is also ideally suited to particle gun-mediated genetic transformation. Instead of relying on *Agrobacterium* to mediate the transfer of genes into plant cells, the particle gun literally shoots DNA that has been precipitated onto particles of a heavy metal, into the plant cells. Embryogenic suspension cultures of cotton and soybean, initiated from immature embryos, yielded an average of 30 stably transformed cell lines following

each firing of the gun. The transformed cell lines can then be induced to form an unlimited number of transformed somatic embryos through repetitive embryogenesis.

Synthesis of metabolites

The repetitive embryogenesis system is of potential use in the synthesis of metabolites such as pharmaceuticals and oils. Borage (*Borage officinalis* L.) seeds contain high levels of ylinolenic acid, used in the treatment of atopic eczema. Somatic embryos of borage also produce this metabolite but through repetitive somatic embryogenesis a continuous supply of y-linolenic acid is ensured, which otherwise would be limited to the growing season in the zygotic embryo. The same principle can be applied for production *in vitro* of industrial lubricants from jojoba (*Simmondsia chinensis*) and leo-palmitostearin (the major ingredient in cocoa butter) from Cacao (*Theobroma cacao*).

The in vivo clonal propagation of plants is often difficult, expensive and even unsuccessful. Tissue culture methods offer an alternative means of plant vegetative propagation. Clonal propagation through tissue culture (popularly called micropropagation) can be achieved in a short time and space. This is possible through somatic embryogenesis by which rapid formation of embryos takes place leading to multiplication of shoots.

Procedure Of Somatic Embryogeneis:

1. Petiole explants plants are surface sterilized and cultured on SH medium (Schenk and Hildebrandt, 1972) containing 2,4-D, kinetin and many other nutrients. 2,4-D activates the cell cycle of many cells in the petiole - those in the vascular cambium develop into a callus, whereas some sub-epidermal cells develop into a somatic embryo.
2. The initial somatic embryos, which are only small dense cell clusters at this stage, are embedded in a callus mass of non-differentiated cells.
3. To liberate these proembryonic structures, and to stimulate the formation of more embryos, the callus is dispersed in a liquid medium to form a suspension culture containing 2,4-D but not kinetin
4. After 7 days, the suspension is sieved and transferred to solid medium lacking 2,4-D On this medium the embryos develop through morphological stages that appear to be globular, heart and torpedo.
5. **Maturation Phase 1**Once the majority of embryos reach the torpedo stage (7-10 days after sieving) they are transferred to an enriched medium containing a high level of sucrose, nitrogen and sulphur to prevent precocious germination and to enable deposition of storage reserves. The embryos rapidly accumulate fresh and dry weight, reaching 1-2 mg dry weight pe
6. **Maturation Phase Ii** To induce the acquisition of desiccation tolerance, the somatic embryos are placed on a modified medium containing abscisic acid (ABA) for 3 days. Then they are removed from the medium, washed to remove sugar and other nutrients, and dried.
7. The standard method of drying is to place the somatic embryos in a sealed chamber over a saturated salt solution designed to give specific relative humidifies. Daily for one week, the embryos are transferred to a progressively lower relative humidity chamber and finally are dried at ambient conditions. At this stage, the embryos have reached approximately 15% moisture and can be stored for a year or more with good viability.

Synthetic seed production

Need for artificial or synthetic seed production technology

Development of micropropagation techniques will ensure abundant supply of the desired plant species. In some crop species seed propagation has not been successful. This is

Box 1. Characteristics of Clonal Propagation Systems

Micropropagation

- Low volume, small scale propagation method
- Maintains genetic uniformity of plants
- Acclimatisation of plantlets required prior to field planting
- High cost per plantlet
- Relatively low multiplication rate

Greenhouse cuttings

- Low volume, small scale propagation method
- Maintains genetic uniformity of plants
- Rooting of plantlets required prior to field planting
- High cost per plantlet
- Multiplication rate limited by mother plant size

Artificial seeds

- High volume, large scale propagation method
- Maintains genetic uniformity of plants
- Direct delivery of propagules to the field, thus eliminating transplants
- Lower cost per plantlet
- Rapid multiplication of plants.

mainly due to heterozygosity of seed, minute seed size, presence of reduced endosperm and the requirement of seed with mycorrhizal fungi association for germination (eg. orchids), and also in some seedless varieties of crop plants like grapes, watermelon, etc. Some of these species can be propagated by vegetative means. However, *in vivo* vegetative propagation techniques are time consuming and expensive. Development of artificial seed production technology is currently considered as an effective and efficient alternate method of propagation in several commercially important agronomic and horticultural crops. It has been suggested as a powerful tool for mass propagation of elite plant species with high commercial value. Characteristics of clonal propagation systems are discussed in Box 1.

Artificial seed technology involves the production of tissue culture derived somatic embryos encased in a protective coating. Artificial seeds have also been often referred to as synthetic seeds. However, the term synthetic seed should not be confused with commercial seeds of a synthetic cultivar which is defined as an advanced generation of an open pollinated population composed of a group of selected inbred clones or hybrids. The concept of artificial or synthetic seed is shown in Figure.

These synthetic seeds would also be a channel for new plant lines produced through biotechnological advances to be delivered directly to the greenhouse or field. Advantages of artificial/ synthetic seeds over somatic embryos for propagation are listed in Box 2. This synthetic seed production technology is a high volume, low-cost production technology. High volume propagation potential of somatic embryos combined with formation of synthetic seeds for low-cost delivery would open new vistas for clonal propagation in several commercially important crop species.

Basic requirement for production of artificial seeds

Recently, production of synthetic seeds by encapsulating somatic embryos has been reported in few species. One prerequisite for the application of synthetic seed technology in micropropagation is the

production of high-quality, vigorous somatic embryos that can produce plants with frequencies comparable to natural seeds. Inability to recover such embryos is often a major limitation in the development of synthetic seeds.

Synthetic seed technology requires the inexpensive production of large numbers of high quality somatic embryos with synchronous maturation. The overall quality of the somatic embryos is critical for achieving high conversion frequencies. Encapsulation and coating systems, though important for delivery of somatic embryos, are not the limiting factors for development of synthetic seeds.

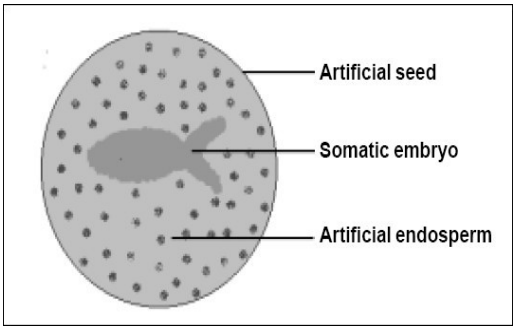
At present, the characteristic lack of developmental synchrony in embryogenic systems stymies multi-step procedures for guiding somatic embryos through maturation. The lack of synchrony of somatic embryos is, arguably, the single most important hurdle to be overcome before advances leading to widespread commercialization of synthetic seeds can occur. Synchronized embryoid development is required for the efficient production of synthetic seeds.

Procedure for production of artificial seeds

Establish somatic embryogenesis
↓
Mature somatic embryos
↓
Synchronize and singulate somatic embryos
↓
Mass production of somatic embryos
↓ Standardization of encapsulation
↓
Standardization of artificial endosperm
↓
Mass production of synthetic seeds
↓
Gr eenhouse and field planting

Box.2. Advantages of Artificial or Synthetic Seeds over Somatic Embryos for Propagation

Ease of handling while in storage
Easy to transport
Has potential for long term storage without losing viability
Maintains the clonal nature of the resulting plants
Serves as a channel for new plant lines produced through biotechnological advances to be delivered directly to the greenhouse or field
Allows economical mass propagation of elite plant varieties.



Types of somatic embryos

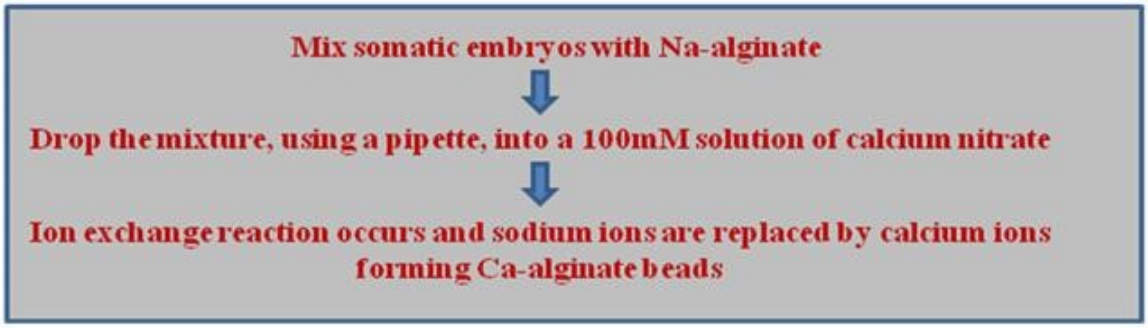
Two types of artificial seeds have been developed, namely, hydrated and desiccated. **Redenbergh et al. (1986) developed hydrated artificial seeds** by mixing somatic embryos of alfalfa, celery and cauliflower with sodium alginate, followed by dropping into a solution of calcium chloride to form calcium-alginate beads. The beads become hardened as calcium alginate is formed. After about 20-30 min. the artificial seeds are removed, washed with water and used for planting. Hydrated artificial seeds are sticky and difficult to handle on a large scale, dry rapidly in the open air. These problems can be resolved by providing a waxy coating over the beads. However, it is not possible to store, except at low temperatures and for limited periods, hydrated artificial seeds and they have to be planted soon after they are produced. Precision machines for large scale encapsulation of SEs have been devised.

Kim and Janick (1989) applied synthetic seed coats to clumps of carrot somatic embryos to develop desiccated artificial seeds. They mixed equal volumes of embryo suspension and 5% solution of polyethylene oxide (polyox WSR N-750), a water-soluble resin, which subsequently dried to form polyembryonic desiccated wafers. The survival of encapsulated embryos was further achieved by embryo hardening treatments with 12% sucrose or10-6M ABA, followed by chilling at high inoculum density.

Desiccated: The desiccated synthetic seeds are produced from somatic embryos either naked or encapsulated in polyoxyethylene glycol (Polyoxr) followed by their desiccation. Desiccation can be achieved either slowly over a period of one or two weeks sequentially using chambers of decreasing relative humidity, or rapidly by unsealing the petri dishes and leaving them on the bench overnight to dry. Such types of synseeds are produced only in plant species whose somatic embryos are desiccation tolerant.

Mix equal volumes of embryo suspension + Polyox (polyoxyethylene)
Suspension was dispensed on to a Teflon sheets (dried suspension sticks to glass plate
Dried to wafers in a laminar flow hood for about 5 h until the wafers get separtaed from teflon plate

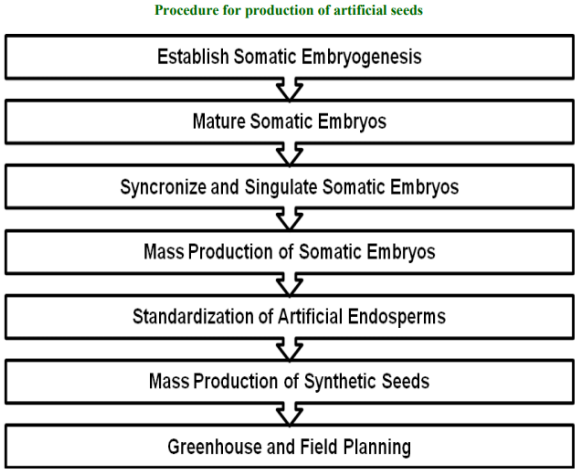
Hydrated: hydrated synthetic seeds are produced in those plant species where the somatic embryos are recalcitrant and sensitive to desiccation. Hydrated synthetic seeds are produced by encapsulating the somatic embryos in hydrogel capsules.


Mix somatic embryos with Na-alginate
Drop the mixture, using a pipette, into a 100mM solution of calcium nitrate
Ion exchange reaction occurs and sodium ions are replaced by calcium ions forming Ca-alginate beads

Procedure For Synthetic Seed Production:

The somatic embryos foe synthetic seeds are produced in the lab through culturing of somatic cells and treating with different hormones to produce root and shoot. The following are the different steps involved in artificial seeds production;

1. Establish somatic embryogenesis
2. Mature somatic embryos
3. Synchronize and singulate somatic embryos
4. Mass production of embryos
5. Encapsulation of matured somatic embryos
6. Desiccation
7. Field planting


Procedure for production of artificial seeds
Establish Somatic Embryogenesis
Mature Somatic Embryos
Syncronize and Singulate Somatic Embryos
Mass Production of Somatic Embryos
Standardization of Artificial Endosperms
Mass Production of Synthetic Seeds
Greenhouse and Field Planning

Another delivery system for somatic embryos for obtaining transgenic plants is Fluid- drilling. Embryos are suspended in a viscous-carrier gel which extrudes into the soil. The primary problem in fluid-drilling is that the sucrose level necessary to permit conversion also promotes rapid growth of contaminating micro-organisms in a non-aseptic system. Gray (1987) found that somatic embryos of orchard grass (*Dactylis glomerata*) became quiescent when desiccated in empty plastic petri dishes at 70% relative humidity at 23°C which amounted to loss of 13% water. However, after 21 days of storage, desiccated embryos when rehydrated *in vitro* germinated to produce viable plantlets though limited (4%) in number. Senaratna *et al* (1990) treated alfalfa somatic embryos with ABA at the torpedo to cotyledonary stages in order to increase their tolerance to desiccation. Over 60% of such desiccated embryos germinated when placed on a moist filter paper or sown directly onto sterile soil and formed plantlets. Heat-shock treatments also induced a degree of desiccation tolerance comparable to that conferred by ABA treatment and had no detrimental effect on the subsequent growth of the plantlets.

In general, the gels contain inorganic nutrients, a carbon source, fungicides, bactericides and other growth promoting substances necessary for SE germination and seedling establishment in the field. When shoot buds are encapsulated, growth regulators needed for rooting and shoot growth are also included. The SE must be of good quality to be able to germinate in a high frequency under field/ greenhouse conditions. Alfalfa is the most extensively investigated plant species in this regard.

Types of gelling agents used for encapsulation

Several gels like agar, **alginate, polyco 2133 (Bordon Co.), carboxyl methyl cellulose, carrageenan, gelrite (Kelko. Co.), guargum, sodium pectate, tragacanth gum,** etc. were tested for synthetic seed production, out of which alginate encapsulation was found to be more suitable and practicable for synthetic seed production. Alginate hydrogel is frequently selected as a matrix for

synthetic seed because of its moderate viscosity and low spin ability of solution, low toxicity for somatic embryos and quick gelation, low cost and bio-compatibility characteristics. The use of agar as gel matrix was deliberately avoided as it is considered inferior to alginate with respect to long term storage. Alginate was chosen because it enhances capsule formation and also the rigidity of alginate beads provides better protection (than agar) to the encased somatic embryos against mechanical injury. Alginate encapsulated somatic embryos of orchids are shown in Figure 1 and the plantlets derived from artificial or synthetic seeds of orchid are shown in Figure 2.

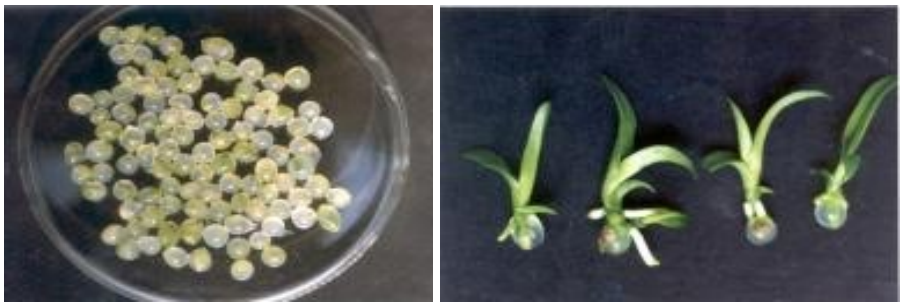


Figure 1 (left). Artificial or synthetic seed produced in orchids by alginate encapsulation. Figure 2 (right). Artificial or synthetic seed derived plantlets in orchid.

Principle and conditions for encapsulation with alginate matrix

Alginate is a straight chain, hydrophilic, colloidal polyuronic acid composed primarily of hydro-□-D-mannuronic acid residues with 1-4 linkages. **The major principle involved in the alginate encapsulation process is that the sodium alginate droplets containing the somatic embryos when dropped into the $CaCl_2.2H_2O$ solution form round and firm beads due to ion exchange between the Na+ in sodium alginate with Ca^{2+} in the $CaCl_2.2H_2O$ solution**. The hardness or rigidity of the capsule mainly depends upon the number of sodium ions exchanged with calcium ions. Hence, the concentration of the two gelling agents i.e., sodium alginate and $CaCl_2.2H_2O$, and the complexing time should be optimized for the formation of the capsule with optimum bead hardness and rigidity. In general, 3% sodium alginate upon complexation with 75 mM $CaCl_2.2H_2O$ for half an hour gives optimum bead hardness and rigidity for the production of viable synthetic seeds.

Artificial endosperm

Somatic embryos lack seed coat (testa) and endosperm that provide protection and nutrition for zygotic embryos in developing seeds. **To augment these deficiencies, addition of nutrients and growth regulators to the encapsulation matrix is desired, which serves as an artificial endosperm. Addition of nutrients and growth regulators to the encapsulation matrix results in increase in efficiency of germination and viability of encapsulated somatic embryos.** These synthetic seeds can be stored for a longer period of time even up to 6 months without losing viability, especially when stored at 4 ^{0}C.

Addition of adjuvants to the matrix

In addition to preventing the embryo from desiccation and mechanical injury, a number of useful materials such as nutrients, fungicides, pesticides, antibiotics and microorganisms (eg. rhizobia) may be incorporated into the encapsulation matrix. **Incorporation of activated charcoal improves the conversion and vigour of the encapsulated somatic embryos**. It has been suggested that charcoal breaks up the alginate and thus increases respiration of somatic embryos (which otherwise lose vigour within a short period of storage). In addition, charcoal retains nutrients within the hydrogel capsule and slowly releases them to the growing embryo.

Utilization of artificial seeds

The artificial seeds can be used for specific purposes, notably multiplication of non-seed producing plants, ornamental hybrids (currently propagated by cuttings) or the propagation of polyploid plants with elite traits. The artificial seed system can also be employed in the propagation of male or female sterile plants for hybrid seed production. Cryo-preserved artificial seeds may also be used for germplasm preservation, particularly in recalcitrant species (such as mango, cocoa and coconut), as these seeds will not undergo desiccation. Furthermore, transgenic plants, which require separate growth facilities to maintain original genotypes may also be preserved using somatic embryos. Somatic embryogenesis is a potential tool in the genetic engineering of plants. Potentially, a single gene can be inserted into a somatic cell. In plants that are regenerated by somatic embryos from a single transgenic cell, the progeny will not be chimeric. Multiplication of elite plants selected in plant breeding programs via somatic embryos avoids the genetic recombination, and therefore does not warrant continued selection inherent in conventional plant breeding, saving considerable amount of time and other resources. Artificial seeds produced in tissue culture are free of pathogens. Thus, another advantage is the transport of pathogen free propagules across the international borders avoiding bulk transportation of plants, quarantine and spread of diseases.

Potential uses of artificial seeds Delivery systems

- Reduced costs of transplants
- Direct greenhouse and field delivery of:

- Elite, select genotypes
- Hand-pollinated hybrids
- Genetically engineered plants
- Sterile and unstable genotypes

- Large-scale mono cultures
- Mixed-genotype plantations
- Carrier for adjuvants such as microorganisms, plant growth regulators pesticides, fungicides, nutrients and antibiotics
- Protection of meiotically-unstable, elite genotypes
- Can be conceivably handled as seed using conventional planting equipment.

Analytical tools

- Comparative aid for zygotic embryogeny
- Production of large numbers of identical embryos
- Determination of role of endosperm in embryo development and germination
- Study of seed coat formation
- Study of somaclonal variation.

Applicability and feasibility of artificial seed production technology

In order to be useful, synthetic seed must either reduce production costs or increase crop value. The relative benefits gained, when weighed against development costs, will determine whether its use is justified for a given crop species. Considering a combination of factors, including improvement of the existing embryogenic systems, relative cost of seed as well as specific application for synthetic seed allows judgement of relative need for a given crop. For example, synthetic seed of seedless watermelon would actually cost less than conventional seed, providing a benefit at the outset of crop production. Although embryogenic systems for this crop do not exist, the benefit that could be conferred by use of synthetic seed would be very great. Value added aspects that would increase crop worth are numerous and include cloning of elite genotypes, such as genetically engineered varieties, that cannot produce true seed.

Questions

1. A plant derived from a somatic embryo is referred as …… a). Seedling b). Embling
   c). Both a and b d). None of the above

2. Somatic embryos are ……
   b).Have apical and basal meristematic regions
   c). Capable of forming shoot and root **c). All the above**

3. Synthetic seeds are of …… types
   a). Hydrated b).Dessicated
   c). Both a and b d). None of the above

4. Hydrated synthetic seeds are developed by ……..
   a). Redenbergh b). Kim and Janick
   c). Both a and b d). None of the above

5. Dessicated synthetic seeds are developed by ……..
   a). Redenbergh **b). Kim and Janick**
   c). Both a and b d). None of the above

6. Hydrated artificial seeds are ……..
   a). Sticky b). Dry rapidly in the open air c). Difficult to handle on a large scale **d). All the above**

7. Gels used for synthetic seed production includes…….. a). Agar and alginate b). Polyco 2133
   c). Carboxy methyl cellulose **d). All the above**

8. Gels used for synthetic seed production includes……..
   a). Carrageenan and gelrite b). Sodium pectate c). Guargum **d). All the above**

9. Alginate hydrogel is frequently selected as a matrix for synthetic seed because of its ……..
   a). moderate viscosity and low spin ability of solution b).low toxicity for somatic embryos and quick gelation
   c). low cost and bio-compatibility characteristics **d). All the above**

10. Gels used for synthetic seed production includes……..
a). Carrageenan and gelrite b). Sodium pectate c). Guargum **d). All the above**

11. Incorporation of activated charcoal in synthetic seed coating improves …….. a). Conversion and vigour of the encapsulated somatic embryos
b). Increases respiration of somatic embryos
c). Retains nutrients within the hydrogel capsule and slowly releases them to the growing embryo
d). All the above

12. The artificial seeds can be used for specific purposes viz., …….. a). Multiplication of non-seed producing plants
b). Propagation of male or female sterile plants for hybrid seed production
c). Propagation of polyploid plants with elite traits
d). All the above

Lecture 11

Production of secondary metabolites

The most important chemicals produced using cell culture are secondary metabolites, which are defined as' those cell constituents which are not essential for survival'. These secondary metabolites include alkaloids, glycosides (steroids and phenolics), terpenoids, latex, tannins etc. It has been observed that as the cells undergo morphological differentiation and maturation during plant growth, some of the cells specialize to produce secondary metabolites. The *in vitro* production of secondary metabolites is much higher from differentiated tissues when compared to non-differentiated tissues.

The cell cultures contribute in several ways to the production of natural products. These are:

- A new route of synthesis to establish products e.g. codeine, quinine, pyrethroids
- A route of synthesis to a novel product from plants difficult to grow or establish e.g. thebain from *Papaver bracteatum*
- A source of novel chemicals in their own right e.g. rutacultin from culture of *Ruta*
- As biotransformation systems either on their own or as part of a larger chemical process e.g. digoxin synthesis.

The advantages of *in vitro* production of secondary metabolites

- The cell cultures and cell growth are easily controlled in order to facilitate improved product formation.
- The recovery of the product is easy.
- As the cell culture systems are independent of environmental factors, seasonal variations, pest and microbial diseases, geographical location constraints, it is easy to increase the production of the required metabolite.
- Mutant cell lines can be developed for the production of novel and commercially useful compounds.
- Compounds are produced under controlled conditions as per the market demands.
- The production time is less and cost effective due to minimal labour involved.

Table showing plant species and secondary metabolites obtained from them using tissue culture techniques

Product	Plant source	Uses
Artemisin	*Artemisia spp.*	Antimalarial
Azadirachtin	*Azadirachta indica*	Insecticidal
Berberine	*Coptis japonica*	Antibacterial, anti

		inflammatory
Capsaicin	*Capsicum annum*	Cures Rheumatic pain
Codeine	*Papaver spp.*	Analgesic
Camptothecin	*Campatotheca accuminata*	Anticancer
Cephalotaxine	*Cephalotaxus harringtonia*	Antitumour
Digoxin	*Digitalis lanata*	Cardiac tonic
Pyrethrin	*Chrysanthemum cinerariaefolium*	Insecticide (for grain storage)
Morphine	*Papaver somniferum*	Analgesic, sedative
Quinine	*Cinchona officinalis*	Antimalarial
Taxol	*Taxus spp.*	Anticarcinogenic
Vincristine	*Cathranthus roseus*	Anticarcinogenic
Scopolamine	*Datura stramonium*	Antihypertensive

Lecture 12
Somaclonal variation and crop improvement

Larkin and Scowcroft (1981) proposed the term somaclone to describe the plants originating from any type of tissue culture. Genetic variation found to occur between somaclones in plant tissue cultures was called somaclonal variation. This variation includes aneuploids, sterile plants and morphological variants, sometimes involving traits of economic importance in case of crop plants. The usefulness of variation was first demonstrated through the recovery of disease resistant plants in potato (resistance against late blight and early blight) and sugarcane (resistance against eye-spot disease, Fiji disease and downy mildew)

Genetic variation - mutations or other changes in the DNA of the tissue those are heritable. This is only transmitted to the next generation and is thus important for crop improvement. Therefore it is necessary to study the transmission of variation to sexual progeny to facilitate the estimation of its utility for improvement of a sexually propagated crop. In several crops R0, R1 and R2 progenies were analyzed for genetic analyses and 3:1 segregation leading to the isolation of true breeding variants was observed.

Epigenetic variation - non-heritable phenotypic variation. Epigenetic changes can be temporary and are ultimately reversible. However, they may also persist through the life of the regenerated plant.

Physiological variation- temporary in response to stimulus and disappear when it is removed.

Causes for variation
Changes of mother plant origin
Chimeral - rearrangement of tissue layers. Many horticultural plants are periclinal chimeras, that is, the genetic composition of each concentric cell layer (LI, LII, LIII) in the tunica of the meristematic tissues is different. These layers can be rearranged during rapid cellular proliferation. Therefore, regenerated plants may contain a different chimeral composition or may no longer be chimera at all. Cell variation also occurs if callus is initiated from explants containing differentiated and matured tissues that have specialized function.
Explant derived variation
The most stable cultures are obtained from meristematic tissue of a mature plant or tissues of a very young organ of meristematic nature. Polyploid cells can give more variability than diploids

Genetic changes arising in culture Ploidy changes
Three phenomena that occur during mitosis lead to most changes in ploidy:

- Endomitosis (sister chromatids separate within the nuclear membrane, but there is no spindle formation nor cytoplasmic division)
- Endoreduplication (chromosomes at interphase undergo extra duplications)
- Spindle fusion (giving binucleate or multinucleate cells).

Gross structural rearrangements appear to be a major cause of somaclonal variation. These involve large segments of chromosomes and so may affect several genes at a time.

- Deletions (genes missing, for example 1,2,3,4 now 1,2,4)
- Inversions (gene order altered, for example 1,2,3,4 now 1,3,2,4)
- Duplications (1,2,3,4 now 1,2,2,3,4)
- Translocations (whole chromosomal segments moved to a new location, for example 1,2,3,4 now 1,2,3,4,A,B,C)

Transposable elements are segments of DNA that are mobile and can insert into coding regions of genes, typically resulting in a lack of expression of the gene. The culture environment may make the transposable elements more likely to excise and move.

Point mutations (the change of a single DNA base), if they take place within a coding region of a gene and result in the alteration of an amino acid, can lead to somaclonal variation. Point mutations are often spontaneous and are more difficult to detect. Note that they result in single gene changes

Structural changes in the DNA sequence
Chromosomal rearrangements, point mutations, or transposition of transposable elements can occur during culture. These changes can occur spontaneously or can be induced with chemicals or radiation

DNA methylation: Most of the mutational events occasioned by tissue culture are directly or indirectly related to alterations in the state of DNA methylation. A decrease in methylation correlates with increased gene activity

Lack of nucleic acid precursors: Shortage of the precursor necessary for rapid nucleic acid biosynthesis, which occurs in many tissue cultures

Growth regulators: One of the triggers of polyploidy *in vitro* is growth regulators; both kinetin and 2,4-D have been implicated.

Composition of culture medium: The level of KNO_3 influences the albino plants from wheat cultures. Level of organic N_2, chelating agents and other micro nutrients are other factors.

Culture conditions: Temperature, method of culture

Effect of the genotype
Effects of the culture process itself (lengthy culture periods, growth and other aspects of the culture medium may also affect the ploidy of the cultured cells. Medium that places cells under nutrient limitation will favor the development of "abnormal" cells. Chromosomal alterations, like ploidy changes, increase with increased lengths of culture. In mixed populations of cells with different ploidy, diploid cells retain their organogenic potential better than polyploid and aneuploid cells (probably due to an enhanced ability to

form meristems).
One common alteration seen in plants produced through tissue culture is *rejuvenation,* especially in woody species. Rejuvenation may lead to changes in morphology, earlier flowering, improved adventitious root formation, and/or increased vigour.

Isolation of somaclonal variants

Mutants for several traits can be far more easily isolated from cell cultures than from whole plant populations. This is because a large number of cells, say 10^6-10^9, can be easily and effectively screened for mutant traits. Screening of as many plants would be very difficult,

ordinarily impossible. Mutants can be effectively selected for disease resistance, improvement of nutritional quality, adaptation of plants to stress conditions, e.g., saline soils, low temperature, toxic metals (e.g., aluminium), resistance to herbicides and to increase the biosynthesis of plant products used for medicinal or industrial purposes. The various approaches to the isolation of somaclonal variants can be grouped into two broad categories: (i) screening and (ii) cell selection.

1. **Screening**

It involves the observation of a large number of cells or regenerated plants for the detection of variant individuals. This approach is the only feasible technique for the isolation of mutants for yield and yield traits. In general, R1 progeny (progeny of regenerated, Ro, plants) are scored for the identification of variant plants, and their R2 progeny lines are evaluated for confirmation. Screening has been profitably and widely employed for the isolation of cell clones that produce higher quantities of certain biochemicals. Computer based automated cell sorting devices have also been used to screen as many as 1000-2000 cells/second from which desirable variant cells were automatically separated.

2. **Cell selection**

In the cell selection approach, a suitable selection pressure is applied which permits the preferential survival/growth of variant cells only. Some examples of cell selection are, selection of cells resistant to various toxins, herbicides, high salt concentration etc. When the selection pressure allows only the mutant cells to survive or divide, it is called positive selection. On the other hand, in the case of negative selection, the wild type cells divide normally and therefore are killed by a counter selection agent, e.g., 5 BUdR or arsenate. The mutant cells are unable to divide as a result of which they escape the counter selection agent. These cells are subsequently rescued by removal of the counter selection agent. Negative selection approach is utilized for the isolation of auxotrophic mutants.

The positive selection approach may be further subdivided into four categories: (i) direct selection, (ii) rescue method, (iii) stepwise selection and (iv) double selection.

In direct selection, the cells resistant to the selection pressure survive and divide to form colonies; the wild type cells are killed by the selection agent. This is the most common selection method. It is used for the isolation of cells resistant to toxins (produced by pathogens), herbicides, elevated salt concentration, antibiotics, amino acid analogues etc.

In the rescue method, the wild type cells are killed by the selection agent, while the variant cells remain alive but, usually, do not divide due to the unfavourable environment. The selection agent is then removed to recover the variant cells. This approach has been used to recover low temperature and aluminium resistant variant cells.

The selection pressure, e.g., salt concentration, may be gradually increased from a relatively low level to the cytotoxic level. The resistant clones isolated at each stage are subjected to the higher selection pressure. Such a selection approach is called stepwise selection. It may often favour gene amplification (which is unstable) or mutations in the organelle DNA.

In some cases, it may be feasible to select for survival and/or growth on one hand and some other feature reflecting resistance to the selection pressure on the other; this is called double selection. An example of double selection is provided by the selection for resistance to the antibiotic streptomycin, which inhibits chlorophyll development in cultured cells. The selection was based on cell survival and colony formation in the presence of streptomycin (one feature) as well as for the development of green colour in these colonies (second feature; only green colonies were selected). This approach has been used for the selection of cells resistant to the herbicide amitrole, 2, 4-D, tobacco mosaic virus (TMV) and aluminium.

Selection of somaclonal variants on subjecting the cells to selection pressure

Selection	Selection of cells in the presence of
Resistance to herbicide	Herbicide
Resistance to salt	Sodium chloride / Aluminium
Resistance to drought	PEG / Mannitol
Resistance to frost	Hydroxy proline resistant lines
Resistance to pathogens	Pathotoxin / Culture filtrate

Crop improvement through somaclonal variation for desirable characters

Crop	Characters modified
Sugarcane	Diseases (eye spot, fiji virus, downy miledew, leaf scald)
Potato	Tuber shape, maturity date, plant morphology, photperiod, leaf colour, vigour, height, skin colour, Resistance to early and late blight
Rice	Plant height, heading date, seed fertility, grain number and weight

Wheat	Plant and ear morphology, awns, grain weight and yield, gliadin proteins,amylase
Maize	T toxin resistance, male fertility, mt DNA
Medicago sativa	Multifoliate leaves, elongated petioles, growth, branch, no.of plants, dry matter yield.
Tomato	Leaf morphology, branching habit, fruit colour, pedicel, male fertility, growth
Avena sativa	Plant height, heading date, awns
Hordeum spp	Plant height and tillering
Lolium hybrids	Leaf size, flower, vigour, survival

Characterization of variants

Somaclonal variants isolated through cell selection are often unstable. The frequency of stable variants may range from 8-62%, perhaps depending on the species and the selection agent. Many selected clones fail to exhibit their resistance during further screening or selection. Obviously these clones are susceptible and were misclassified as resistant, called as **escapes**. Several clones lose their resistance to the selection agent after a period of growth in the absence of selection pressure. Such clones are called unstable variants and may result from changes in gene expression and from gene amplification (increase in the number of copies of a gene per genome of the organism in comparison to that naturally present). Some variant phenotypes are quite stable during the cell culture phase, but they disappear when plants are regenerated from the variant cultures, or when the regenerated plants reproduce sexually, in case they are expressed in the regenerated plants. Such changes are known as **epigenetic changes** and are attributed to stable changes in gene expression e.g., hormone habituation of cell cultures and, possibly, cold resistance in *Nicotiana sylvestris*.

The remaining variants which stably express the variant phenotypes during the cell culture as well the regenerated plant phases, and exhibit the transmission of these phenotypes through the sexual reproduction cycle are called mutants. Only this category of variants would find an application in crop improvement. These may represent true gene mutations or some other types of changes. Usually, expected mendelian ratios are obtained in the Rl progenies. But sometimes aberrant segregation ratios are encountered in Rl possibly due to the chimeric nature of Ro plants, the involvement of some cytological anomalies like aneuploidy, deletions etc., gene dosage effects etc.

Achievements

Over a dozen varieties have been developed through the exploitation of somaclonal variation. ‘Ono’ variety of sugarcane is a Fiji disease resistant somaclone of the susceptible cultivar ‘Pindar’. It was identified by screening of plants regenerated from unselected calli. ‘Ono’ also shows yield advantage over ‘Pindar’ and has been cultivated to

a limited extent in Fiji. A sweet potato cultivar 'Scarlet' was selected from shoot-tipculture-derived clones. 'Scarlet' is comparable to the parent cultivar in yield and disease resistance, but shows darker and more stable skin colour, which is a desirable quality trait. A geranium variety called 'Velvet Rose' is a somaclone of 'Rober's Lemon Rose'. The new variety has twice the chromosome number of the parent variety. An alfalfa variety called 'Sigma' is a polycross of selected somaclones.

In India, so far somaclonal variation is the only biotechnological approach to give a commercial variety. A somaclonal variant of Citronella java, a medicinal plant, has been released as 'Bio-13' for commercial cultivation by CIMAP (Central Institute for Medicinal and Aromatic Plants), Lucknow. Bio-13 yields 37% more oil and 39% more citronellol than the control varieties. A somaclonal variant of the B. juncea variety 'Varuna' has been released for commercial cultivation as 'Pusa Jai Kisan'. The new variety has bolder seeds and some yield advantage over the parent variety Varuna.

Advantages

- Somaclonal variations occur in rather high frequencies, which is a great advantage over conventional mutagenesis.
- Some 'new' alleles or even 'new' mutations may be isolated which were not available in the germplasm or through mutagenesis, e.g., joint less pedicel mutant in tomato.
- Use of somaclonal variation may reduce by two years the time required for the release of new variety as compared to mutation breeding. This is because somaclonal variations are usually free from undesirable features like sterility, while induced mutations are generally associated with such defects, which necessitate one or two backcrosses with the parent variety.
- A very effective selection can be practised at the cell level for several traits, e.g., disease resistance etc. This approach effectively selects few desirable cells from among millions with relatively small effort, time, cost and space requirements.
- This is the only approach for the isolation of biochemical mutants, especially auxotrophic mutants, in plants.

Limitations

- The technique is applicable only to those species of cell cultures which regenerate complete plants.
- Selected cell lines often show reduced or no regeneration potential.
- Many selected clones show undesirable features like reduced fertility, growth and even overall performance.

Somaclonal variation represents a useful source of introducing genetic variations that could be of value to plant breeders. Single gene mutation in the nuclear or organelle genome may give the best available variety in vitro that has a specific improved character. In this manner, somaclonal variation could be used to uncover new variants retaining all the favourable characters along with an additional useful trait, such as resistance to diseases or a herbicide. Various cell lines selected in vitro may then prove potentially applicable to agriculture and industry.

Questions

1. The term somaclone was proposed by ……
   a). Larkin and Scowcroft b). Skoog
   c). Murashige d). None of the above

2. Somaclonal variation includes ……
   a). Aneuploids b). Sterile plants c). Morphological variants **d). All the above**

3. The usefulness of somaclonal variation was first demonstrated through the recovery of disease resistant plants in ……
   a). Potato b). Sugarcane
   c). Both a and b d). None of the above

4. Epigenetic variation includes ……
   a). Non-heritable phenotypic variation b). Temporary
   c). Ultimately reversible **d). All the above**

5. Ploidy changes occur due to ……. during mitosis
   a). Endomitosis b). Endoreduplication
   c). Spindle fusion **d). All the above**

6. Gross structural rearrangements viz., ……. are major cause of somaclonal variation
   a). Deletions and duplications b). Inversions
   c). Translocations **d). All the above**

7. Ono is a somaclonal variety of …….
   a). Sugarcane b). Geranium
   c). Alfa alfa d). Citronella

8. Scarlet is a somaclonal variety of …….
   a). Sugarcane **b). Sweet potato**
   c). Alfa alfa d). Citronella

9. Velvet rose is a somaclonal variety of …….
   a). Sugarcane **b). Geranium**
   c). Alfa alfa d). Citronella

10. Sigma is a somaclonal variety of …….
    a). Sugarcane b). Geranium
    c). Alfa alfa d). Citronella

11. Bio 13 is a somaclonal variety of …….
    a). Sugarcane b). Geranium
    c). *Brassica juncea* **d). Citronella**

12. Varuna is a somaclonal variety of …….

a). Sugarcane b). Geranium

c). *Brassica juncea* d). Citronella

13. Limitation(s) of somaclonal variation is/are …….

a). Applicable only to those species which regenerate complete plants out of cell cultures

b). Reduced or no regeneration potential

c). Undesirable features like reduced fertility, growth and even overall performance

d). All the above

Lecture 13

Germplasm conservation

1. Introduction

In recent years, with the enormous increase in the population, pressure on the forest and the land resources have increased. This results in depletion of population of medicinal and aromatic plant species. Even some of the plant species are at the verge of vanishing from the forest. The list of endangered species is growing day by day. The conventional methods of germplasm preservation are prone to possible catastrophic losses because of:

- i. Attack by pathogen and pests.
  ii. Climatic disorders
  iii. Natural disasters and
  iv. Political and economic causes

In addition, the seeds of many important medicinal plants lose their viability in a short time under conventional storage system.

The conservation of germplasm can be done by two methods:

1. In-situ preservation: Preservation of the germplasm in their natural environment by establishing biospheres, national parks etc.

2. Ex-situ preservation: In the form of seeds or by *in vitro* cultures.

Seeds form the most common material to conserve plant germplasm, however, the method has the following disadvantages:

- i. Discrete clones cannot be maintained in the form of seeds.
  ii. Some plants do not produce fertile seeds.
  iii. Loss of seed viability.
  iv. Seed destruction by pests, etc.
  v. Poor germination rate.
  vi. This is useful for seed propagating plants and is not applicable to vegetatively propagated crops, like potato, ginger etc.

Cryopreservation (Greek, karyos - frost)

Cryopreservation means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell and tissue cultures to zero metabolism or non-dividing state by mean of storage of germplasm at a very low temperatures, (i) Over solid CO_2 (-79°C), (ii) Deep freezers (-80°C), (iii) in vapor phase nitrogen (-150°C), (iv) in liquid nitrogen (-196°C).

Among these, the most commonly used non-lethal storage of biological material at ultra-low temperature is by employing liquid nitrogen. At the temperature of liquid nitrogen (-196°C), almost all the metabolic activities of cells are ceased and the sample can then be preserved in such state for extended periods.

Steps involved in cryopreservation

The technique of cryopreservation involves the following steps:

1. Selection of plant material
2. Pre-culture
3. Cryoprotective treatment
4. Freezing and storage
5. Thawing
6. Reculture

Questions:

1. The methods involved in the *in vitro* conservation of germplasm are ……...
   a). Cryopreservation b). Cold storage
   c). Low pressure and low oxygen storage **d). All the above**
2. Cryopreservation involves the usage of ……...
   a). Solid carbon dioxide (at -79^{0}C) b). Vapour nitrogen (at- 150^{0}C)
   c). Liquid nitrogen (at-196^{0}C) **d). All the above**
3. The major benefits of synthetic seeds are ……...
   a). Easy to store without viability loss b). Easy to handle
   c). Can be directly sown in soil **d). All the above**
4. Tissue culture is used for ……
   a). Vegetative multiplication b). Virus-free plants
   c). Both a and b d). None of the above
5. Tissue culture has potential application in ……
   a). Production of somatic hybrids b). Organelle and cytoplasm transfer
   c). Genetic transformation **d). All the above**
6. Virus free plants in tissue culture are produced by ……..
   a). Meristem tip culture b). Shoot tip culture
   c). Nodal culture d). All the above
7. Tissue culture is used for …..
   a). Production of virus free plants b). Rejuvenation of old plant materials
   c). Hybrid production **d). All the above**
8. Cryo preservation of tissue culture materials in liquid nitrogen is at
   a). –196^0 C b). –190^{0}C c). – 96^0 C d). –90^{0}C
9. Dimethylsufoxide is used as …… in tissue culture.
   a). Cryoprotectant b). Growth regulator
   c). Osmaticum d). None of the above